UFSM

DOCTOR OF PHILOSOPHY THESIS

INTERACTION OF ORGANODISELENIDES WITH SULPHYDRYL GROUPS AT THE ACTIVE SITES OF SOME THIOL CONTAINING PROTEINS - *IN VITRO* AND *IN VIVO* MECHANISTIC STUDIES IN MAMMALIAN MODELS OF DIABETES

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Santa Maria, RS, Brazil

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A PhD THESIS SUBMITTED TO THE POSTGRADUATE PROGRAMME IN BIOCHEMICAL TOXICOLOGY, CENTRE FOR NATURAL AND EXACT SCIENCES FEDERAL UNIVERSITY OF SANTA MARIA, RS BRAZIL, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD) IN BIOCHEMICAL TOXICOLOGY

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The undersigned Examining Committee, approved the Doctor of Philosophy Thesis entitled

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Presented by Ige Joseph KADE in partial fulfillment of the requirements for the award of the degree of doctor of philosophy (PhD) in Biochemical Toxicology

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Dedication

To the Source of Light and Life and the Creator of Science

Jehovah God

Also to the King Designate and Redeemer of Mankind from Diseases

Jesus Christ

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Prof. Joao Batista Teixeira da Rocha

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Mrs Oluwayomi Peace Faromika.

Thanks for allowing Jehovah God to use you just at the right time to save life.

To others who have in one way or the other been there for me always, I say thanks and best wishes to you all.

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Organization of the Thesis

The results that make up this thesis are presented in form of written manuscripts and are found under the scientific articles. The sections **Materials and Methods, Results, Discussion** of Results and **References** are found in the Articles itself and represent an integral part of this study.

The items, **Discussion** and **Conclusions** found at the beginning of this thesis are general interpretation and comments on the scientific articles presented.

The **References** refers only to the citations that appears in the items **Introduction**, **Literature Review, Discussion and Conclusions** of this thesis

Resumo Geral

O presente estudo quis comparar os potenciais antioxidants *in vitro* de organoselênios novamente sintetizados, diseleneto dicolesterol e diseleneto de difenila e suas possíveis interações com algumas enzimas contendo tióis em diferentes tecidos de mamíferos. Além disso, o potencial de DPDS como agente antioxidante e antihiperglicêmico, e sua interação com proteínas contendo tióis em vários tecidos e órgãos de mamíferos (hepático, renal, esplênico e, mais importante, tecido cerebral) foram avaliados em modelos animais de streptozotocina induzindo diabetes em ratos. Os resultados *in vitro* mostram que DPDS exibiu uma maior atividade mimética da glutationa peroxidase bem como aumentada habilidade para oxidar mono e di-tióis que DPDS. Além disso, enquanto o DPDS inibiu substâncias reativas ao ácido tiobarbitpúrico (TBARS) e formação de proteínas carboniladas em tecidos cerebral e hepático, induzidas por ferro(II) ou SNP, DCDS exibiu um efeito pró-oxidante em cérebro e tecido hepático quando ferro(II) serviu como pró-oxidante, porém, quando TBARS foi induzido por SNP, DCDS modificou a formação de TBARS tanto em tecido cerebral como hepático. Também, as atividades da delta-aminolevulinato desidratase (ð-ALA-D) cerebral e hepática e Na^+/K^+-ATPase cerebral foram significativamente inibidas por DPDS e somente fracamente inibida por DCDS. Mas estudos revelam que a inibição causada por organodiselenetos (neste caso, DPDS) na atividade da Na^+/K^+-ATPase envolve a modificação de grupos tiólicos ligados ao sítio ATP da enzima. Similarmente, diferentes isoformas da lactato desidrogenase (LDH) foram significativamente inibidas por DPDS e DCDS *in vitro*. nós observamos que a inibição *in vitro* de diferentes isoformas da LDH por DCDS e DPDS envolve a modificação de grupos –SH no sítio ligante NAD+ da enzima. a administração oral de DPDS dissolvido em óleo soya administrado a ratos albino machos com diabetes induzida por streptozotocina mostrou que houve uma redução significante nos níveis de glicose sanguínea acompanhada

por uma marcada redução nas proteínas glicadas em ratos diabéticos induzidos com streptozitocina tratados com DPDS em relação aos não diabéticos. Além disso, DPDS melhorou significativamente os níveis de vitamina C e GSH (fígado, rim e baço), que foram diminuídos em ratos tratados com streptozotocina. Similarmente, tratamento com DPDS marcadamente aboliu os níveis elevados de TBARS que foram observados no grupo diabético. Finalmente, a inibição da ð-ALA-D e algumas isoformas da LDH causada pela hiperglicemia foram prevenidas por DPDS. Nós também observamos que STZ provocou uma significante diminuição no status antioxidante do cérebro e atividade da Na^+/K^+-ATPase, mas a atividade da acetilcolinesterase e captação e liberação de glutamato não foram alteradas. Porém, DPDS marcadamente restaurou o desequilíbrio observado no status antioxidante e bomba de sódio. Finalmente, nós concluímos que organoselenetos são remédios antioxidantes promissores no manejo de doenças causadas por estresse oxidativo. Porém, sua toxicidade envolve uma interação com tióis em proteínas e este estudo demonstrou que os grupos sulfidril em questão são críticos para a função normal de enzimas e proteínas. Estes –SH são associados com tióis dos sítios de ligação do substrato (sítio ativo) de enzimas. interessantemente, doses farmacológicas de organodiselenetos (3mg/kg para o estudo de diabetes) não apresentou nenhuma toxicidade observada.

General Abstract

The present study sought to compare the *in vitro* antioxidant potentials of a newly synthesized organodiselenide, dicholesteroyl diselenide (DCDS) and diphenyl diselenide (DPDS) and their possible interactions with some thiol containing enzymes in different tissues from mammalian system. In addition, the potency of DPDS as antioxidant and antihyperglycaemic agents, and its interaction with thiol containing proteins in various mammalian tissues and organs (hepatic, renal and spleenic and more importantly cerebral tissues) were evaluated in animal models of streptozotocin induced diabetic rats.

The *in vitro* results show that DPDS exhibited a higher glutathione-peroxidase mimetic activity as well as increased ability to oxidize both mono- and di- thiols than DCDS. In addition, while DPDS inhibited thiobarbituric acid reactive substances (TBARS) and protein carbonyls formations in both cerebral and hepatic tissues, induced by either iron (II) or SNP, DCDS exhibited a prooxidant effect in both cerebral and hepatic tissues when iron (II) serves as the prooxidant, However, when TBARS was induced by SNP, DCDS slightly modify TBARS formation in both hepatic and cerebral tissues. Also the activities of cerebral and hepatic delata aminolevulinic acid dehydratase (ð-ALA-D), cerebral Na^+/K^+-ATPase were significantly inhibited by DPDS and only weakly inhibited by the DCDS. Further studies reveal that the inhibition caused by organodiselenides (in this case, DPDS) on Na^+/K^+-ATPase activity likely involves the modification of the thiol groups at the ATP binding site of the enzyme. Similarly, different isoforms of lactate dehydrogenase (LDH) were significantly inhibited by both DPDS and DCDS *in vitro*. Likewise, we observed that the *in vitro* inhibition of different isoforms of lactate dehydrogenase by DCDS and DPDS likely involves the modification of the -SH groups at the NAD^+ binding site of the enzyme.

Oral administration of DPDS dissolved in soya bean oil administered to streptozotocin induced diabetes in male albino rats shows that there was significant reduction in blood

glucose levels accompanied by a marked reduction of glycated proteins in streptozotocin induced diabetic rats treated with DPDS in relation to untreated streptozotocin induced diabetic. In addition, DPDS was able to significantly ameliorate the levels of Vitamin C and GSH (liver, kidney and spleen), which were decreased in streptozotocin treated rats. Similarly, treatment with DPDS was able to markedly abolish the increase levels of TBARS that were observed in STZ diabetes group. Finally, the inhibition of both ð-ALA-D and some isoforms of LDH caused by hyperglycaemia were prevented by DPDS. We also observed that although streptozotocin evoke a significant diminution on brain's antioxidant status and activity of Na^+/K^+-ATPase, but the activity of acetylcholineesterase and glutamate uptake and release were not altered. However, DPDS was able to markedly restore the observed imbalance in antioxidant status and sodium pump.

Finally, we conclude that organodiselenides are promising antioxidant remedy in the management of diseases caused by oxidative stress. However, their toxicity involves an interaction with thiols on proteins and this study has further demonstrated that the sulphydryl groups in question are critical to the normal function of the protein or enzymes. Most likely, these -SH are associated with thiols at the substrate binding (active site) sites of the enzymes. Interestingly, pharmacological doses of organodiselenides 3mg/kg bw for the study on diabetes do not present any observed toxicity.

1.0 Introduction

A major landmark in biological science is the understanding that the etiologies of many diseases are consequent of an imbalance in the antioxidant systems. Consequently, this has evoked intensified efforts in the search for drugs that can restore these imbalances caused by free radicals in degenerative diseases. Generally these drugs conventionally referred to as antioxidants can either be synthetic or of natural origin. Interestingly, compounds of elements in groups 13 have been shown to possess antioxidative properties. Particularly, much attention has been give to the promising role of selenium compounds as candidate remedy for degenerative diseases. However, although selenium compounds are pharmacologically promising, they are also very toxic. In fact, excellent reviews (Mugesh *et al*., 2001, Nogueira *et al.,* 2004) have related the toxicity of selenium compounds to their ability to interact with thiols of biochemical and physiological importance on proteins. Therefore, unraveling the precise mechanisms of interaction of selenium compounds with thiols on proteins has continued to be a dilemma to the biological and chemical scientists. Consequently, this has evoked continued interest at understanding and elucidation of the precise mechanism of interaction of organoselenium compounds with critical sulphydryl groups on biologically important proteins both at the cellular and molecular levels.

Diphenyl diselenide belongs to the diorganyl diselenide class of organoselenium compounds. While it has been demonstrated that diphenyl diselenide interacts with thiols and consequently inhibits thiol-containing enzymes such as delta aminolevulinic acid dehydratase (Barbosa *et al*., 1998) and Na^+/K^+- ATPase (Borges *et al*., 2005), there is a dearth of information on the possible location of these critical thiol groups that interact with selenium compounds. Hence there is need to continue to design experiments aimed at understanding precise mechanism involved in such interactions.

Additionally, selenium compounds possess differential toxicity (Tinggi *et al*., 2003) which critically depends on animal species and vehicle solution. Hence, since Barbosa *et al*., (2006) recently reported that diphenyl diselenide possess antihyperglycaemic activites when administered subcutaneously and in Tween solution, it is worthwhile investigating if the antihyperglycaemic property of diphenyl diselenide is altered when administered via a different routes and vehicle solutions. Therefore, taken together, we observed from the foregoing that a lot of puzzling questions are yet to be resolved vis a vis pharmacology and toxicology of organochalcogens. This then necessitated continuous concerted investigations that would provide excellent explanations to the puzzles related to the chemistry of selenium compounds that has remained an enigma for decades. This work therefore partly serves as a contribution to the unraveling of the chemistry of organoselenium compounds.

1.1 Free Radicals and the Physiological System

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals (Halliwell and Gutteridge, 1999). This unpaired electron(s) usually gives a considerable degree of reactivity to the free radical. Radicals derived from oxygen represent the most important class of radical species generated in living systems (Miller, *et al*., 1990). Molecular oxygen (dioxygen) has a unique electronic configuration and is itself a radical. The addition of one electron to dioxygen forms the superoxide anion radical ($O_2^{\bullet -}$) (Miller *et al*., 1990) (Fig. 1 shows some pathways of free radical formation). Superoxide anion, arising either through metabolic processes (mostly in mitochondria) or following oxygen "activation" by physical irradiation, is considered the "primary" reactive oxygen species (ROS), and can further interact with other molecules to generate "secondary" ROS, either directly or prevalently through enzyme- or metal-catalysed processes (Valko, *et al*., 2005). During energy

transduction, a small number of electrons "leak" to oxygen prematurely, forming the oxygen free radical superoxide, which has been implicated in the pathophysiology of a variety of diseases (Valko *et al*., 2004).

Aside from $O_2^{\bullet-}$, hydroxyl radical, $^{\bullet}OH$ is another important ROS with high reactivity, making it a very dangerous radical with a very short *in vivo* half-life of approx. 10^{-9} s and thus reacts close to its site of formation (Pastor, *et al*., 2000). The redox state of the cell is largely linked to an iron (and copper) redox couple and is maintained within strict physiological limits. However, *in vivo*, under stress conditions, an excess of superoxide releases "free iron" from iron-containing molecules such as from the [4Fe–4S] cluster containing enzymes of the dehydratase-lyase family (Liochev and Fridovich, 1994). The released Fe^{2+} can participate in the Fenton reaction, generating highly reactive hydroxyl-radical ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$). Thus under stress conditions, $O_2^{\bullet-}$ acts as an oxidant of [4Fe–4S] cluster-containing enzymes and facilitates $^{\bullet}OH$ production from H_2O_2 by making Fe^{2+} available for the Fenton reaction (Valko *et al*., 2005; Leonard *et al*., 2004). The superoxide radical participates in the Haber–Weiss reaction ($O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^-$) which combines a Fenton reaction and the reduction of Fe^{3+} by superoxide, yielding Fe^{2+} and oxygen ($Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$) (Liochev and Fridovich, 2002). *In vivo,* production of $^{\bullet}OH$ according to the Fenton reaction occurs when $metal^{n+}$ is iron, copper, chromium, or cobalt. Additional reactive radicals derived from oxygen that can be formed in living systems are peroxyl radicals ($ROO^{\bullet}$), such as hydroperoxyl or perhydroxyl radical $HOO^{\bullet}$, which is the protonated form of ($O_2^{\bullet-}$) making up only about 0.3% of any ($O_2^{\bullet-}$) present in the cytosol of a typical cell (De Grey, 2002). The $HOO^{\bullet}$ initiates fatty acid peroxidation by two parallel pathways: fatty acid hydroperoxide (LOOH)-independent and LOOH-dependent (Aikens and Dix, 1991).

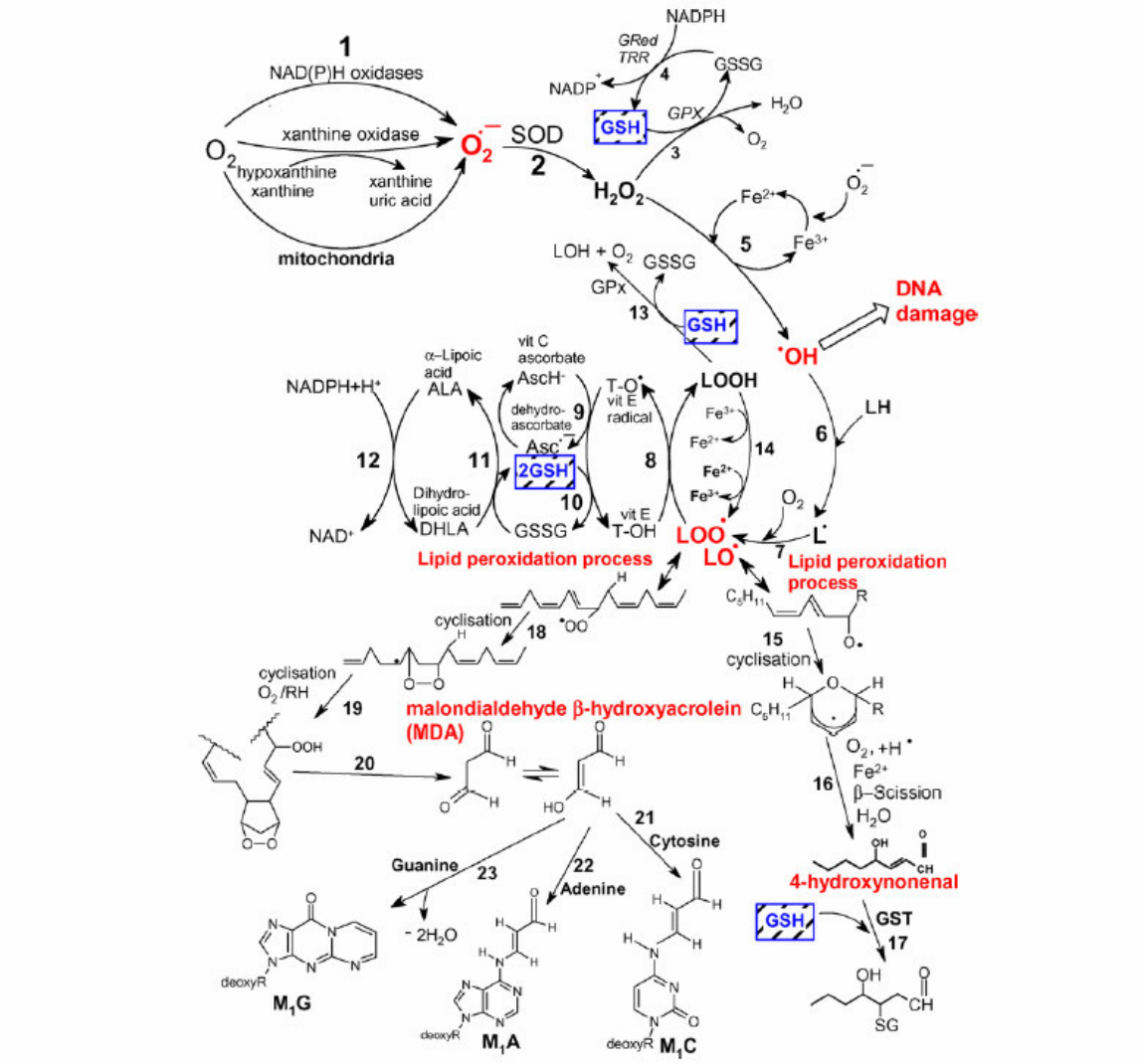


Figure 1: *Pathways of ROS formation, the lipid peroxidation process and the role of glutathione (GSH) and other antioxidants (Vitamin E, Vitamin C, lipoic acid) in the management of oxidative stress (equations are not balanced). Reaction 1: The superoxide anion radical is formed by the process of reduction of molecular oxygen mediated by NAD(P)H oxidases and xanthine oxidase or non-enzymatically by redox-reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain. Reaction 2: Superoxide radical is dismutated by the superoxide dismutase (SOD) to hydrogen peroxide. Reaction 3: Hydrogen peroxide is most efficiently scavenged by the enzyme glutathione peroxidase (GPx) which requires GSH as the electron donor. Reaction 4: The oxidised glutathione (GSSG) is reduced back to GSH by the enzyme glutathione*

reductase (Gred) which uses NADPH as the electron donor. Reaction 5: Some transition metals (e.g. Fe2+, Cu+ and others) can breakdown hydrogen peroxide to the reactive hydroxyl radical (Fenton reaction). Reaction 6: The hydroxyl radical can abstract an electron from polyunsaturated fatty acid (LH) to give rise to a carbon-centred lipid radical (L•). Reaction 7: The lipid radical (L•) can further interact with molecular oxygen to give a lipid peroxyl radical (LOO•). If the resulting lipid peroxyl radical LOO• is not reduced by antioxidants, the lipid peroxidation process occurs (reactions 18–23 and 15–17). Reaction 8: The lipid peroxyl radical (LOO•) is reduced within the membrane by the reduced form of Vitamin E (T-OH) resulting in the formation of a lipid hydroperoxide and a radical of Vitamin E (T-O•). Reaction 9: The regeneration of Vitamin E by Vitamin C: the Vitamin E radical (T-O•) is reduced back to Vitamin E (T-OH) by ascorbic acid (the physiological form of ascorbate is ascorbate monoanion, AscH−) leaving behind the ascorbyl radical (Asc•−). Reaction 10: The regeneration of Vitamin E by GSH: the oxidised Vitamin E radical (T-O•) is reduced by GSH. Reaction 11: The oxidised glutathione (GSSG) and the ascorbyl radical (Asc•−) are reduced back to GSH and ascorbate monoanion, AscH−, respectively, by the dihydrolipoic acid (DHLA) which is itself converted to _-lipoic acid (ALA). Reaction 12: The regeneration of DHLA from ALA using NADPH. Reaction 13: Lipid hydroperoxides are reduced to alcohols and dioxygen by GPx using GSH as the electron donor. Lipid peroxidation process: Reaction 14: Lipid hydroperoxides can react fast with Fe2+ to form lipid alkoxyl radicals (LO•), or much slower with Fe3+ to form lipid peroxyl radicals (LOO•). Reaction 15: Lipid alkoxyl radical (LO•) derived for example from arachidonic acid undergoes cyclisation reaction to form a six-membered ring hydroperoxide. Reaction 16: Six-membered ring hydroperoxide udergoes further reactions (involving_-scission) to from 4-hydroxy-nonenal. Reaction 17: 4-hydroxynonenal is rendered into an innocuous glutathiyl adduct (GST, glutathione S-transferase). Reaction 18 A peroxyl radical located in the internal position of the fatty acid can react by cyclisation to produce a cyclic peroxide adjacent to a carbon-centred radical. Reaction 19: This radical can then either be reduced to form a hydroperoxide (reaction not shown) or 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. Reaction 20: Formed compound is an intermediate product for the production of malondialdehyde. Reactions 21, 22, 23: Malondialdehyde can react with DNA bases Cytosine, Adenine, and Guanine to form adducts M1C, M1A and M1G, respectively. (Valko et al., 2007)

The LOOH-dependent pathway of $HO_2^{\bullet-}$-initiated fatty acid peroxidation may be relevant to mechanisms of lipid peroxidation initiation *in vivo*. Peroxisomes are known to produce H_2O_2, but not $O_2^{\bullet-}$, under physiologic conditions (Valko *et al*., 2004). In peroxisomes antioxidant enzymes such as catalase maintains a delicate balance to ensure no net production of ROS. When peroxisomes are damaged, their H_2O_2 consuming enzymes down regulate H_2O_2 releases into the cytosol which is significantly contributing to oxidative stress. Moreso, xanthine oxidase (XO, EC 1.1.3.22) and xanthine dehydrogenase (XD, EC 1.1.1.204) are interconvertible forms of xanthine oxidoreductase (XOR) (Vorbach, *et al*., 2003) and catalyze the oxidative hydroxylation of hypoxanthine to xanthine and subsequently of xanthine to uric acid. Uric acid acts as a potent antioxidant and free radical scavenger. XOR has, therefore, important functions as a cellular defense enzyme against oxidative stress. However, with both XO and XD forms, but particularly with the XO form, numerous ROS and RNS are synthesized (Vorbach *et al*., 2003). Thus, the synthesis of both an antioxidant (uric acid) and numerous free radicals (ROS and RNS) makes XOR an important protective regulator of the cellular redox potential.

In addition to ROS, reactive nitrogen species (RNS) such as $NO^{\bullet}$ (containing one unpaired electron on the antibonding $2\pi_y^*$ orbital) are also 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 and Cadenas, 2005). Nitric oxide (NO•) is an abundant reactive radical that acts as an important oxidative biological signaling molecule in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defense mechanisms, smooth muscle relaxation and immune regulation (Bergendi, *et al*., 1999). However, in the extracellular milieu, $NO^{\bullet}$ reacts with oxygen and water to form nitrate and nitrite anions.

Overproduction of reactive nitrogen species is called nitrosative stress (Ridnour *et al*., 2004). This may occur when the generation of RNS 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. In addition, 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, *et al*., 2000): $\{NO^\bullet + O_2^{\bullet-} \rightarrow ONOO^-\}$. Also, $NO^\bullet$ readily binds certain transition metal ions; such as binding to Fe^{2+}-Haem groups in the enzyme soluble guanylate cyclase (sGC) (Archer, 1993). $Fe^{2+}\{sGC\} + NO^\bullet \rightarrow Fe^{2+}\{sGC\}–NO$ (2)

The product can either be $\{Fe^{2+}–NO^\bullet\}$ or $\{Fe^{3+}–NO^-\}$.

Hence, we observed from the abovementioned that both ROS and RNS are products of normal cellular metabolism playing dual roles as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems (Valko, *et al*., 2006). Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular responses to noxia, as for example in defense against infectious agents and in the function of a number of cellular signalling systems. The harmful effect of free radicals causing potential biological damage is termed oxidative stress and nitrosative stress (Kovacic and Jacintho, 2001; Ridnour *et al*., 2005). This occurs in biological systems when there is an overproduction of ROS/RNS on one side and a deficiency of enzymatic and non-enzymatic antioxidants on the other. In other words, oxidative stress results from the metabolic reactions that use oxygen and represents a disturbance in the equilibrium status of prooxidant/antioxidant reactions in living organisms. The excess ROS can damage cellular lipids, proteins, or DNA inhibiting their normal function. Because of this, oxidative stress has been implicated in a number of human diseases as well as in the ageing process.

1.2 Free Radicals and Cellular Macromolecular Damage

As mentioned above, both RNS and ROS at high concentration can be important mediators of damage to cell structures, nucleic acids, lipids and proteins (Valko *et al*., 2006). The hydroxyl radical is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone (Halliwell and Gutteridge, 1999). Permanent modification of genetic material resulting from these "oxidative damage" incidents represents the first step involved in mutagenesis, carcinogenesis, and ageing. It is known that metal-induced generation of ROS results in an attack not only on DNA, but also on other cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation (Siems, *et al*., 1995). Once formed, peroxyl radicals ($ROO^{\bullet}$) can be rearranged *via* a cyclisation reaction to endoperoxides (precursors of malondialdehyde) with the final product of the peroxidation process being the mutagenic and carcinogenic compound, malondialdehyde (MDA) (Wang *et al*., 1996) (Fig. 1). Aside from MDA, other lipid peroxidation product is 4-hydroxy- 2-nonenal (HNE) a weakly mutagenic but major toxic product of lipid peroxidation.

Mechanisms involved in the oxidation of proteins by ROS were elucidated by studies in which amino acids, simple peptides and proteins were exposed to ionizing radiations under conditions where hydroxyl radicals or a mixture of hydroxyl/superoxide radicals are formed (Stadtman, 2004). The side chains of all amino acid residues of proteins, in particular cysteine and methionine residues of proteins are susceptible to oxidation by the action of ROS/RNS (Stadtman, 2004). Oxidation of cysteine residues may lead to the reversible formation of mixed disulphides between protein thiol groups (–SH) and low molecular weight thiols, in particular GSH (*S*-glutathiolation). The concentration of carbonyl groups, generated by many different mechanisms is a good measure of ROS-

mediated protein oxidation. A number of highly sensitive methods have been developed for the assay of protein carbonyl groups (Dalle-Donne *et al*., 2005; Dalle-Donne, *et al*., 2003). Advanced glycation end products (AGEs) is a class of complex products. They are the results of a reaction between carbohydrates and free amino group of proteins. The intermediate products are known, variously, as Amadori, Schiff Base and Maillard products, named after the researchers who first described them (Dalle-Donne *et al*., 2005). Most of the AGEs are very unstable, reactive compounds and the end products are difficult to be completely analysed. The brown colour of the AGEs is probably related to the name of melanoidins initially proposed by Maillard, and well known from food chemistry. The best chemically characterized AGEs compounds found in human are pentosidine and carboxyl methyl lysine (CML).

1.3. Free Radicals and Antioxidants

Exposure to free radicals from a variety of sources has led organisms to develop a series of defence mechanisms (Cadenas, 1997). Defence mechanisms against free radical-induced oxidative stress involve: (i) preventative mechanisms, (ii) repair mechanisms, (iii) physical defences, and (iv) antioxidant defenses. Enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT). Non-enzymatic antioxidants are represented by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants. 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. Glutathione is a tripeptide, a thiol antioxidant and redox buffer of the cell which is highly abundant in the cytosol (1–11 mM), nuclei (3–15 mM), and mitochondria (5–11 mM) and is the major soluble antioxidant in these cell compartments. Recently, it has been

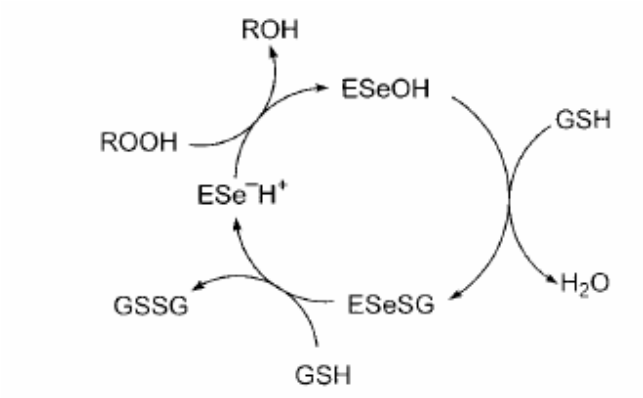
shown that externally added GSH is readily taken up by mitochondria, despite the ~8mM GSH present in the mitochondrial matrix (Shen, *et al*., 2005). It therefore appears that GSH is taken up against a concentration gradient. GSH in the nucleus maintains the redox state of critical protein sulphydryls 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 (Nogueira, *et al*., 2004; Jones *et al*., 2000). Too high a 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 possibly by preventing glycation of such amino acids; (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) (Pastore, *et al*., 2003). 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 a number of papers (El-Agamey *et al*., 2004; Kojo, 2004; Miller *et al*., 2005; Smith, *et al*., 2004).

1.4 Organoselenium as Antioxidants

It is evident that Se has multiple roles in biological systems. Many of them reside in its capability of acting as an antioxidant and disease preventing element. Excellent reviews (Nogueira *et al*., 2004, Mugesh *et al*., 2001) have shown that organoselenium compounds are much less toxic compared with the inorganic selenium species. Hence a lot of effort has been directed toward the development of stable organoselenium compounds that could be used as antioxidants, enzyme inhibitors, antitumor and anti-infective agents, cytokine inducers, and immunomodulators (Nogueira *et al*., 2004, Mugesh *et al*., 2001). A number of synthetic organoselenium compounds are known to act as antioxidants by reducing H_2O_2 and ONOO and also by preventing lipid peroxidation. The antioxidant properties of organoselenium compounds have been related to their glutathione peroxidase (GPx) mimetic activity, their ability to reduce peroxynitrite and prevent lipid peroxidation.

1.4.1 Organoseleniums Exhibit Glutathione Peroxidase Mimetic Activity

Since the demonstration that Ebselen exhibit glutathione peroxidase mimetic activity (GPx), (Muller *et al*., 1984) several groups have worked toward the design and synthesis of new GPx mimics by modifying the basic structure of ebselen or by incorporating some structural features of the native enzyme. This has led to the design of diorganyl diselenide compounds such as diphenyl diselenide which exhibits moderate GPx activity. As mentioned above, RNS are produced as part of normal metabolic processes. However, the inhibition of superoxide and nitric oxide by ebselen in rat Kupffer cells sparked interest in other synthetic organoselenium compounds as to their potential function in scavenging peroxyl nitrite (PN) (Wang, 1992).

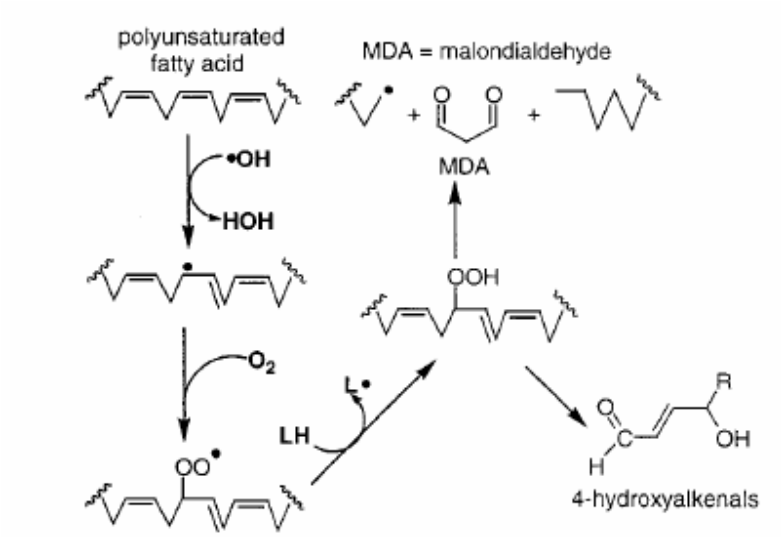


Scheme 2: Glutathione peroxidase mimics of selenium compounds (Mugesh et al., 2001).

In fact, organoselenides, such as ebselen protected DNA from single-strand break formation caused by peroxynitrite more effectively than its sulfur analogue (Masumoto, 1996).

1.5 Lipid Peroxidation

Polyunsaturated fatty acids are particularly susceptible targets of ROS attack. Abstraction of a hydrogen atom from a polyunsaturated fatty acid initiates the process of lipid peroxidation (Figure 3). In the third step of Figure 3, a hydrogen atom is abstracted from a second lipid, leading to a new ROS. Numerous lipid peroxidation products are formed that can react with sulfhydryl (cysteine) or basic amino acids (histidine, lysine) (Sies, 1986). 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 3: Scheme for lipid peroxidation (Mugesh et al., 2001)

1.6 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.

1.7 Organoseleniums Inhibit Thiol Enzymes

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-S-transferase (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 antiinflammatory 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.

1.7.1 Delta aminolevulinic acid dehydratase

Delta-aminolevulinate dehydratase or porphibilinogen synthase (ð -ALA-D) is a sulfhydryl-containing enzyme that is extremely sensitive to oxidizing agents (Rocha, *et al*., 1995). ð-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 (Figure 4). 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.

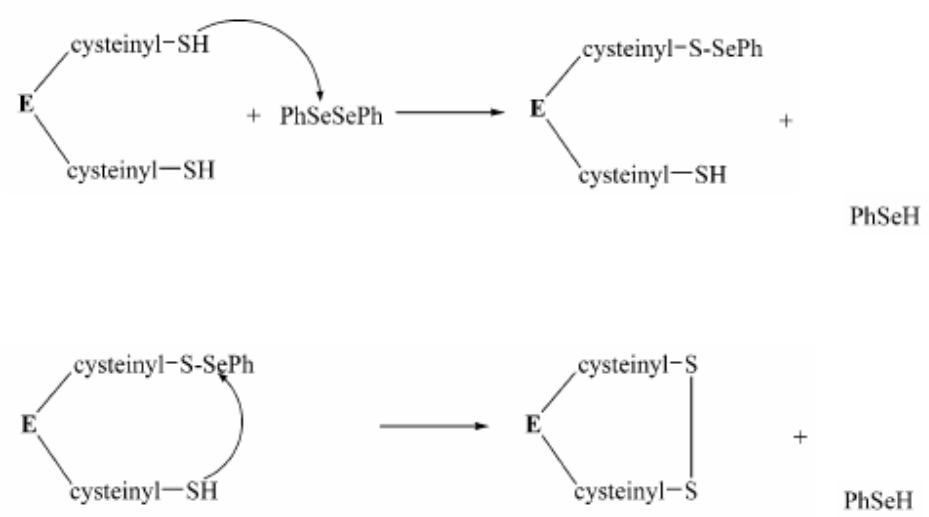


Figure 4: Possible mechanism of interaction of organodiselenides with thiol on proteins (Nogueira *et al*., 2004)

1.7.2 Na/K pump –principle of function

Na^+/K^+-ATPase is the largest protein complex in the family of P-type cation pumps. The P-ATPase name comes from the observation of a transient phosphorylation during the functional cycle: the γ-phosphate of ATP is transferred to an aspartate residue (D376). At rest it consumes 20–30% of ATP production to actively transport Na^+ out of and K^+ into the cell thus maintaining cell's membrane potentials, volume, and secondary active transport of other solutes, e.g., the transcellular transport processes in intestine, glands, and kidney. The minimum functional unit is a heterodimer of the α- and β-subunits (Jorgensen and Andersen, 1988). The P-ATPases have also been named E_1–E_2 ATPases because they can be found in two main conformations, E_1 and E_2, which differ in many aspects. In the case of the Na-K-ATPase, for which the most extensive studies have been performed in this regard, the E_1 and E_2 conformations differ, for instance, in affinity for Na^+ and K^+, sensitivity to ADP and ATP, intrinsic fluorescence, and sensitivity to proteolysis.

1.7.2.1 Na^+ and K^+ binding sites: The Na-K-ATPase provide a binding site to accommodate three Na^+ ions according to its well-accepted stoichiometry of 3 Na^+/2 K^+/1 ATP. In a detailed analysis of the Na-K-ATPase model, Ogawa and Toyoshima (2002) propose that two sodium ions occupy two sites homologous to the calcium sites I and II of SERCA and predict the position of a third sodium site at about the same level in the membrane but closer to transmembrane segment 9. The three-dimensional structure of these cation binding sites and the contribution of the fourth, fifth, sixth, eighth, and ninth transmembrane helices have been described in literature (Ogawa and Toyoshima, 2002; Toyoshima and Inesi, 2004).

1.7.2.2 Binding sites for extracellular inhibitors: Some of these inhibitors are largely used in clinical medicine: the cardiac steroid inhibitors of the Na-K-ATPase are

used for the treatment of heart failure (the pharmacological target is most probably the α 2-isoform of the Na-K-ATPase), and the gastric proton pump inhibitors are used for the treatment of peptic ulcers. It has long been known that the substituted benzimidazole compounds that are used for proton pump inhibition act by forming a covalent bond, a disulfide bridge, with one (or two) specific cysteine residues located in the sixth transmembrane segment of the α-subunit (Lambrecht *et al*., 1998). The binding site of the cardiac steroid on the Na/K pump has been more difficult to define. Comparison of ouabain-sensitive and -resistant isoforms of the Na/K pump and subsequent mutagenesis work (Lingrel *et al*., 1994) initially pointed to the outer part of the first hairpin (the transmembrane segments 1 and 2 and the short extracellular loop between them), but extensive mutagenesis work disclosed the important contribution of the fifth, sixth, and seventh transmembrane segments (Palasis *et al*., 1996), and recent work with Na-K-ATPase and H-K-ATPase chimerical constructs demonstrated that the third hairpin (transmembrane segments 5 and 6) provides the structure of the binding site itself (Qiu, *et al*., 2003). The very strong influence of the extracellular part of the first and second transmembrane segments can be understood by the hypothesis that this domain may modulate the binding on or off rate by acting on the access to the ouabain-binding site, as suggested by recent work comparing the kinetics of ouabain binding in human α-isoforms (Crambert *et al*., 2004).

1.7.2.3 Lipid–protein interactions with the Na^+,K^+-ATPase: Lipid dynamics are essential to enzymatic function (Cornelius, 2001). Anionic phospholipids are necessary for efficient reconstitution (Cornelius and Skou, 1984). A minimum number of phospholipids must be retained to support full activity of the solubilised enzyme (Esmann, 1984). Free fatty acids, which display a selectivity for the Na,K-ATPase, which also specifically inhibit

the enzyme (Ahmed and Thomas, 1971), whereas phosphatidylserine stabilises the solubilised enzyme (Hayashi *et al*., 1989; Shinji *et al*., 2003). Hence the precise fascinating biophysical dynamics of lipid–protein interactions in cellular membrane are crucial to the functioning of Na^+/K^+-ATPase.

1.7.3 Lactate dehydrogenase

Lactate dehydrogenase (LDH, EC 1.1.1.27) exists as five tetrameric isozymes including combinations of two different types of subunits. The two types of subunits H (heart) and M (muscle) form H4, M4, H3M, H2M2 and HM3, have different catalytic, physical and immunological properties. Lactate dehydrogenase (LDH) (Lang-Unnasch and Murphy, 1998) the a key enzyme in the glycolytic pathway is a 2-hydroxy acid oxidoreductase that converts pyruvate to lactate and simultaneously the conversion of NADH to NAD^+. As a constant supply of NADH is a prerequisite for glycolysis, and LDH acts as the primary source for the regeneration of NADH from NAD^+, inhibition of LDH is expected to stop production of ATP, with subsequent cell death. Therefore, any compound that blocks the LDH enzyme has been suggested as potentially potent anti bacterial drugs and can be employed in malarial treatment (Cameron *et al*., 2004) As a case study, *P. falciparum* lactate dehydrogenase (LDH) has been suggested as a drug target (Gomez *et al.,* 1997). One well recognized difficulty is that the drug must potently inhibit other baterial LDH yet show much less activity against the three human LDH isoforms. For example, a comparison of the crystal structures of both *P. falciparum* and human LDH (Read *et al*., 2001; Dunn *et al*., 1996) shows the following two key differences: namely positioning of the NADH factor, reflecting sequence changes in the cofactor binding pocket that displace the nicotinamide ring by about 1.2 Å, and a change in the sequence (including a 5-residue insertion) and secondary structure of a loop region that closes down on the active site

during catalysis. These changes combine to produce an increase in the volume of the active site cleft in *plasmodium falciparum* LDH (*pf*LDH) relative to its human counterparts. In addition, to structural variations, isoforms of LDH exhibits significant kinetic differences (Makler *et al*., 1993). Taken together, the structural and kinetic discrepancies between the isoforms of LDH suggest that different and specific, but potent inhibitors of varied isoforms of LDH can be designed or identified. In fact, several groups are known to have targeted isoforms of *P. falciparum* (*pf*LDH) in drug discovery studies (Gomez *et al.,* 1997; Yu *et al*., 2001; Dando *et al.,* 2001). The continued search for drugs with selective inhibitory potency on the different isoforms of LDH have continued to be an attractive venture.

1.8 Diabetes and Reactive Oxygen Species

Diabetes mellitus can be classified as either type 1, or insulin dependent diabetes (IDD) or type 2 or non insulin dependent diabetes (NIDD) (Brownlee and Cerami, 1981; Niedowicz and Daleke, 2005). NIDD is most common form of diabetes. Decreased uptake of glucose into muscle and adipose tissue leads to chronic extracellular hyperglycemia resulting in tissue damage and pathophysiological complications, involving heart disease, atherosclerosis, cataract formation, peripheral nerve damage, retinopathy and others (Brownlee and Cerami, 1981). Increased oxidative stress has been proposed to be one of the major causes of the hyperglycemia-induced trigger of diabetic complications. Hyperglycemia in an organism stimulates ROS formation from a variety of sources. These sources include oxidative phosphorylation, glucose autooxidation, NAD(P)H oxidase, lipooxygenase, cytochrome P450 monooxygenases, and nitric oxide synthase (NOS).

1.8.1 Sources of ROS in diabetes: Under normal conditions, the key sites of superoxide formation in the mitochondrial membrane are complex I and the ubiquinone–complex III interface, where the presence of long lived intermediates allows reaction of electrons with molecular dioxygen (Kwong and Sohal, 1998). However, diabetes alters the primary sites of superoxide generation so that complex II becomes the primary source of electrons that contribute to superoxide formation under diabetic conditions (Nishikawa *et al*., 2000). This conclusion comes from the study of a complex II inhibitor, 2-thenoyltrifluoroacetone and an uncoupler of oxidative phosphorylation, carbonyl cyanide *m*chlorophenyldihydrazone leading to a decrease in ROS formation in various cells exposed to high concentration of glucose (Yamagishi, *et al*., 2001). Another source of ROS in diabetes is NAD(P)H oxidase. Several lines of evidence support that NAD(P)H oxidases are a major source of glucose induced ROS production in the vasculature and kidney cells, confirming thus NAD(P)H as a mediator of diabetic complications (Li and Shah, 2003). Involvement of other cells has not been satisfactorily confirmed. Since hypertension is a common complication of diabetes, it is possible that expression of NAD(P)H oxidase is regulated similarly in both these disease states. This arises from increased angiotensin II labelling in cardiac myocytes and endothelial cells from human diabetic patients (Li & Shah, 2003). High glucose-induced formation of ROS and p47phox (cytosolic component of activated NAD(P)H) can be blocked with AngII type 1 receptor antagonists, confirming thus a link between the two pathways of NAD(P)H oxidase activation. The NAD(P)H oxidase-mediated production of ROS in diabetes can be suppressed by a variety of PKC inhibitors, implicating this family of kinases in the regulation of hyperglycemia-induced NAD(P)H oxidase activity. Since hyperglycemia-induced oxidative stress occurs in non nucleated cells lacking mitochondria and the NAD(P)H oxidase (erythrocytes), another mechanism of ROS formation in such cells must exist. A possible explanation for such

behaviour is glucose auto-oxidation (Robertson, *et al*., 2003). Glucose itself, as well as its metabolites, is known to react with hydrogen peroxide in the presence of iron and copper ions to form hydroxyl radical. Evidence for this comes from *in vitro* experiments and therefore *in vivo* studies should be carried out.

In addition to ROS, RNS have been implicated as one of the sources of nitrosative stress in diabetes. $NO^{\bullet}$ can react with superoxide forming peroxynitrite, a highly reactive oxidant linked with many disease states including diabetes (Zou, *et al*., 2002). Peroxynitrite reacts with the zinc-cluster of NOS leading to its uncoupling, suggesting that peroxynitrite not only depletes exisiting $NO^{\bullet}$ but also reduces a tissue's ability to produce more $NO^{\bullet}$. Xanthine oxidase (XO) has been proposed to be a major source of ROS in diabetes mellitus (Butler, *et al*., 2000). Treatment of non-insulin dependent diabetes patients with the XO inhibitor allopurinol reduces the level of oxidised lipids in plasma and improves blood flow. Experimental data suggests that the role for XO is tissue-dependent. Lipooxygenases catalyse conversion of arachidonic acid into a broad class of signalling molecules, such as leukotrienes, lipoxins, and hydroxyeicosatetraenoic acid. Diabetes is associated with increased lipoxygenase expression, resulting in eicosanoid formation (Brash, 1999). Production of ROS and RNS depletes both enzymatic and non-enzymatic antioxidants leading to additional ROS/RNS accumulation causing cellular damage. Vitamin E is depleted in diabetes and has a protective effect mainly through suppressed lipid peroxidation. Vitamin C levels in plasma have also been found to be reduced in diabetes patients; however, the relation between reduced levels of Vitamin C and diabetic complications is unclear (VanderJagt, *et al*., 2001).

The effect of diabetes on glutathione (GSH) peroxidase activity is highly variable with respect to the model of diabetes and type of tissue used. The data suggest hyperglycemia and diabetes complications affect the regulation of GSH peroxidase

expression; however the effect to which GSH peroxidase inhibition affects cell health is unclear (VanderJagt *et al*., 2001). Hyperglycemic treatment impaired no change in activity of GSH reductase, thus it does not appear that GSH reductase plays a role in the onset of diabetic complications. Various consequences of oxidative stress in diabetic subjects involve accumulation of MDA. However, the role for 4-hydroxy-nonenal in diabetes is not yet clear; a few studies have reported the accumulation of 4- HNE and the activation of signalling pathways (Traverso *et al*., 2002). Isoprostanes, non-enzymatic products of arachidonic acid oxidation, have been found to be elevated in diabetic rats and in plasma and urine of noninsulin dependent patients. The data are suggestive, but do not definitively support an active role for isoprostanes in the onset of diabetic complications. Isoprostanes have become popular markers of lipid peroxidation in diabetes and other disease, in part because of their specificity and sensitivity of detection. Glucose can react directly with free amine groups on protein and lipids, finally yielding a diverse group of modifications referred as advanced glycation end products (AGE) (Ling *et al*., 2001). AGEs are observed primarily in long-lived structural proteins, such as collagen. AGEs are found in almost all tissues examined from diabetic rats and in human non-insulin dependent patients. Some tissues, such as liver, kidneys, and erythrocytes are more susceptible to AGE formation than others. Thus AGE formation is probably a significant contributor to the onset of diabetic complications, mainly atherosclerosis.

2.0 Objectives

2.1 Main Objective

The main objective of this work is to elucidate the possible critical thiol groups on proteins that interact with organodiselenides as well as provide a possible explanation on the effect of non-selenium moiety of diorganoyl diselenium compounds on their chemistry in both *in vitro* and *in vivo* mammalian models of diabetes.

2.2 Specific Objectives

The specific objectives of this work are:

1. To compare the *in vitro* antioxidant effect of diphenyl diselenide DPDS and a novel organodiselenide, dicholesteroyl diselenide (DCDS) under iron (II) and sodium nitroprusside induced cerebral and hepatic TBARS production.

2. To compare the *in vitro* inhibitory effect of both DPDS and DCDS on the activity some thiol containing enzymes: delta aminolevulinic acid dehydratase (ð-ALA-D), Na^+,/K^+ ATPase, and different isoforms of Lactate dehydrogenase (LDH)

3. To investigate the possible modulation of antioxidant levels and the activity of thiol containing proteins such as ð-ALA-D, and LDH in diabetic rats administered orally with DPDS previously dissolved in soya bean oil.

4. To investigate the *ex vivo* interaction of DPDS and STZ with thiol containing enzymes in STZ induced diabetic rat models as well as their interaction with some neuronal processes.

3.0 SCIENTIFIC MANUSCRIPTS

3.1 *In Vitro* Studies

3.1.1 First Manuscript

COMPARATIVE STUDIES ON DICHOLESTEROYL DISELENIDE AND DIPHENYL DISELENIDE AS ANTIOXIDANT AGENTS AND THEIR EFFECT ON THE ACTIVITIES OF Na+/K+-ATPase AND ð-AMINOLEVULINIC ACID DEHYDRATASE IN THE RAT BRAIN

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ORIGINAL PAPER

Comparative Studies on Dicholesteroyl Diselenide and Diphenyl Diselenide as Antioxidant Agents and their Effect on the Activities of Na^+/K^+ ATPase and δ-Aminolevulinic acid Dehydratase in the Rat Brain

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Abstract The present study sought to evaluate the effect of a newly synthesized selenium compound, dicholesteroyl diselenide (DCDS) and diphenyl diselenide (DPDS) on the activities of delta-aminolevulinate dehydratase and Na^+/K^+-ATPase in the rat brain. The glutathione peroxidase mimetic activity of the two compounds as well as their ability to oxidize mono- and di- thiols were also evaluated. The antioxidant effects were tested by measuring the ability of the compounds to inhibit the formation of thiobarbituric acid reactive species and also their ability to inhibit the formation of protein carbonyls. The results show that DPDS exhibited a higher glutathione peroxidase mimetic activity as well as increased ability to oxidize di-thiols than DCDS. In addition, while DPDS inhibited the formation of thiobarbituric acid reactive species and protein carbonyls, DCDS exhibited a prooxidant effect in all the concentration range (20–167 μM) tested. Also the activities of cerebral delta-aminolevulinate dehydratase and Na^+/K^+ ATPase were significantly inhibited by DPDS but not by DCDS. In addition, the present results suggested that the inhibition of Na^+/K^+ ATPase by organodiselenides, possibly involves the modification of the thiol group at the ATP binding site of the enzyme. In conclusion, the results of the present investigation indicated that the non-selenium moiety of the organochalcogens can have a profound effect on their antioxidant activity and also in their reactivity towards SH groups from low-molecular weight molecules and from brain proteins.

Keywords Organoselenium compounds · Na^+/K^+ ATPase · ∂-ALA-D · TBARS · GSHPx · Protein carbonyls

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Introduction

There is increasing evidence that the mammalian brain may be exceptionally vulnerable to oxidative stress through oxygen radical attack which possibly contribute to cerebral ischemic injury by promoting membrane lipid peroxidation and oxidative damage to DNA and proteins [1–5]. In fact, several reports have implicated free radical overproduction in the etiology of a variety of acute and chronic neurodegenerative situations [5–7]. However, clinically effective drugs for the treatment of these diseases are rare. Consequently, continued efforts geared towards the development and biological testing of new antioxidant compounds for the treatment of these neurological disorders have increased considerably in recent times [8–12].

Reports have shown that these selenium-containing organic compounds are generally more potent antioxidants than classical antioxidants and this fact serves as an impetus for an increased interest in the rational design of synthetic organoselenium compounds [13, 14]. The development of these organoselenium compounds is based on their ability to mimic the antioxidant enzyme glutathione peroxidase (GSHPx) activity in vitro. The glutathione peroxidase -mimetic of organoselenium compounds,

which, like the native enzyme, rely on the redox cycling of selenium, have been reported in the literature [13, 15]. In addition, a recent report described the thioredoxin reductase and thioredoxin peroxidase mimetic activity of organoselenides and related these effects as another possible mechanism for their observed antioxidant actions [16].

Diphenyl diselenide is a synthetic organodiselenide with potent antioxidant potentials [15, 17–19]. Earlier reports have indicated that DPDS affects a number of neuronal processes [14, 20, 21]. Nogueira et al. reported that DPDS increases the basal activity of adenylyl cyclase and inhibits [^{3}H]glutamate, [^{3}H]MK-801, and unstable [^{3}H] guanylylimidodiphate binding to rat synaptic membrane preparations after both in vitro and ex vivo exposure [10]. Further study reveals that DPDS effectively reduced ^{45}Ca influx into isolated nerve endings when a nondepolarizating condition was used or when 4-aminopyridine (4-AP) was used as a depolarizing agent [15]. Recently DPDS was also reported to inhibit the cerebral Na^+, K^+-ATPase in a concentration dependent manner and that the inhibition may occur through a change in the crucial thiol groups of this enzyme [22].

There are strong points of evidence suggesting that modification of the organic moiety of organoselenium compounds can have a profound effect on their biological activity [15, 23]. On the other hand, there are reports indicating that compounds containing the steroidal molecules could possess pharmacological potency and that small alterations in the structure of these steroids can greatly affect their receptor binding affinity and biological activity [24]. In fact, several studies have demonstrated that the modulation of the various heterocyclic rings of various steroids was effective in the production of a variety of compounds possessing potent biological activities [25, 26]. These observations aforementioned prompted the synthesis of a novel organoselenide compound in the organic synthesis unit of our laboratory. This novel compound possesses the cholesterol steroidal molecule as the organic moiety component.

However, earlier observations [27, 28] have demonstrated that chronic exposure to high doses of diselenides may cause central effects in animal models, suggesting that the brain may be a potential target for the toxic effect of organoselenium compounds. In addition, recent reports from our laboratory have indicated that DPDS has proconvulsant effect in mice model, an effect that can be further related to its neurotoxicity [23, 29]. However, the mechanism(s) involved in the neurotoxicity of DPDS is still unknown. Although DPDS possess antioxidant activity in vitro and in vivo including neuroprotective effects [30], it can also be neurotoxic. One can speculate that the neuroprotective effect of organochalcogens may be related to their antioxidant activity and that its neurotoxic activity may be related to its ability to oxidize thiol groups in enzymes. Therefore, the present study was aimed at comparing the in vitro antioxidant potential of this novel compound, DCDS with that of DPDS in the rat brain. In addition, the possible inhibitory effects of these two compounds on the thiol containing enzymes from brain namely cerebral delta aminolevulinic acid dehydratase and Na^+/K^+-ATPase were also evaluated. In fact, the main objective of this study was to investigate how changes in the non-selenium moiety of organochalcogens could modify their reactivity towards thiols and also whether their antioxidant properties could be modified by these changes. Furthermore, we also carried out a detailed study on the possible protective effect of ligands and substrate on the inhibitory effect of DPDS on brain Na^+,K^+-ATPase.

Experimental procedure

Chemicals

DPDS and DCDS (Scheme 1) were synthesized according to literature methods [31]. These drugs were dissolved in 99% ethanol. Analysis of the ^{1}HNMR and ^{13}CNMR spectra showed that all the compounds obtained presented analytical and spectroscopic data in full agreement with their assigned structures. The purity of the compounds were assayed by high resonance mass spectroscopy (HRMS) and was higher that 99.9%. All chemicals used were of analytical grade and obtained from Sigma–Aldrich, FLUKA, BDH and other standard commercial suppliers. ATP (sodium salt) was purchased from Sigma–Aldrich Co.

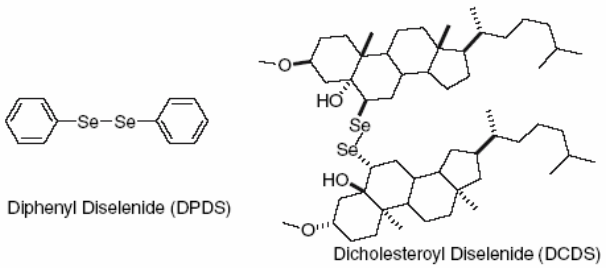
Animals

Male adult Wistar rats (200–250 g) from our own breeding colony were used. Animals were kept in separate animal cages, on a 12-h light: 12-h dark cycle, at a room temperature of 22–24°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 RS, Brazil.

Preparation of tissue homogenate for thiobarbituric acid reactive species (TBARS) assay

Rats were decapitated under mild ether anesthesia and the cerebral tissue (whole brain) was rapidly removed, placed on ice and weighed. Tissues were immediately homogenized in cold 10 mM Tris–HCl, pH 7.4 (1/10, w/v) with 10 up-and-down strokes at approximately 1,200 rev/min in a Teflon-glass homogenizer. The homogenate was

Scheme 1 Structure of diphenyl diselenide and cholesteroyl diselenide



centrifuged for 10 min at 4,000*g* to yield a pellet that was discarded and a low-speed supernatant (S1). An aliquot of 100 μl of S1 was incubated for 1 h at 37°C in the presence of both organodiselenides (final concentrations range of 21–167 μM), with and without the prooxidants; iron (final concentration(10 μM) and sodium nitroprusside (SNP) (final concentration 3 μM). This was then used for lipid peroxidation determination. One rat brain was used per experiment. Production of TBARS were determined as described by method of Ohkawa et al. [32] excepting that the buffer of color reaction have a pH of 3.4. The color reaction was developed by adding 300 μl 8.1% SDS to S1, followed by sequential addition of 500 μl acetic acid/HCl (pH 3.4) and 500 μl 0.8% of thiobarbituric acid (TBA). This mixture was incubated at 95°C for 1 h. TBARS produced were measured at 532 nm and the absorbance was compared to that of a standard curve obtained using malondialdehyde (MDA).

Effect of organodiselenides on protein carbonyls formation

Brain was homogenized in a proportion of 1:20 in 10 mM Tris–HCl (pH 7.4). Homogenates were then incubated in the presence of organodiselenides (concentration range 20–80 μM) and iron (5 μM) and control groups were also prepared. The tubes were incubated for 18 h. After incubation, the protein carbonyls determination was carried out as described by Reznick and Packer [33] with some modifications. Briefly, three 0.8-ml brain homogenate samples were placed in glass tubes. Thereafter, 0.2 ml of 2,4-dinitrophenylhydrazine (DNPH) 10 mM in 2.0 M HCl was added to two of the tubes while the other tube contains only 0.2 ml of 2.0 M HCl solution (blank for DNPH-independent A365, to correct the background absorbances in brain samples). Tubes were incubated for 60 min at room temperature, in the dark. Samples were vortexed every 15 min. Then, 0.5 ml of denaturizing buffer (sodium phosphate buffer, pH 6.8, containing SDS 3%), 1.5 ml of ethanol and 1.5 ml of hexane were added, the mixture was vortexed for 40 s and centrifuged for 5 min at 2,000 rpm. The pellet obtained was separated and washed 3 times with 2 ml ethanol: ethyl acetate (1:1, v/v). The precipitate was dissolved in 1 ml denaturizing buffer solution and was incubated for 10 min at 37°C with mixing. Any insoluble material was removed by additional centrifugation. Carbonyl content was calculated from the peak absorbance of the spectra at 355–390 nm, using an absorption coefficient of 22,000 M^{-1} cm^{-1}. Protein was measured by the method of Lowry et al. [34], using bovine serum albumin as standard. Results were expressed as nmol carbonyl/mg of protein.

Thiol peroxidase activity

The catalytic effects of the organodiselenides on the reduction of H_2O_2 by reduced glutathione were assessed using the rate of glutathione (GSH) oxidation. Free –SH groups were determined according to Ellman [35]. DPDS and DCDS at concentration range of 50–150 μM were incubated in the medium containing GSH (5.0 mM) with and without H_2O_2 (2 mM) at 2.5 mins intervals for 15 min. Aliquots of the reaction mixture (200 μl) were checked for the amount of GSH at the indicated time intervals.

Oxidation of mono and di-thiols

The rate of thiol oxidation was determined in the presence of 50 mM Tris–Cl, pH 7.4, and 250 μM of organodiselenides. The rate of thiol oxidation was evaluated by measuring the disappearance of –SH groups. Free –SH groups were determined according to Ellman [35]. Incubation at 37°C was initiated by the addition of the thiol compounds. Aliquots of the reaction mixture (100 μl) were checked for the amount of –SH groups at 412 nm after 90–120 min of addition of color reagent 5'5'-dithio-bis(2-nitrobenzoic) acid (DTNB).

ð-Aminolevulinate dehydratase (ð-ALA-D) activity

Cerebral ð-ALA-D activity was assayed according to the method of Sassa [36] by measuring the rate of product

porphobilinogen (PBG) formation except that 100 mM potassium phosphate buffer and 2.5 mM ∂-ALA were used. Rats cerebral were homogenized in Tris–HCl in the proportion 1:3 (w/v) and centrifuged at 4,000*g* for 15 min. An aliquot of 100 μl of supernatant (S1) was incubated for 3 h at 37°C. Reaction was linear in relation to protein and time of incubation. The reaction product was determined using modified Erlich's reagent at 555 nm.

Effect of organodiselenides on Na^+, K^+-ATPase activity

Immediately after the sacrifice, the brain was removed and the homogenate was prepared in 0.05 M Tris–HCl, pH 7.4, sucrose buffer (pH 7.4). The homogenate was centrifuged at 2,000*g* at 4°C for 7 min and supernatant was again centrifuged at 12,000*g* for 10 min. The pellets (P2) were reconstituted in the homogenizing buffer to give a final protein concentration of 5 to 6 mg/ml and used for assay of Na^+, K^+-ATPase. The reaction mixture for Mg^{2+}-dependent-Na^+, K^+-ATPase activity assay contained 3 mM MgCl, 125 mM NaCl, 20 mM KCl and 50 mM Tris–HCl, pH 7.4 and 100–120 μg of protein, in a final volume of 500 μl. The reaction was initiated by addition of ATP to a final concentration of 3.0 mM. Controls were carried out under the same conditions with the addition of 0.1 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays. Released inorganic phosphorous (Pi) was measured by the method of Fiske and Subbarow [37].

To check whether pre-incubation of homogenates without a cationic component of the assay medium will affect the interaction of organodiselenides with the Na^+/K^+-ATPase activity, organodiselenides and enzyme (P2) were incubated at 37°C for 10 min, with the selective exclusion of each Mg^{2+}, Na^+, K^+ in the preincubating medium. All the experiments were conducted at least three times and similar results were obtained. Protein was measured by the method of Lowry et al. [34], using bovine serum albumin as standard.

For all enzyme assays, incubation times and protein concentration were chosen to ensure the linearity of the reactions. All samples were run in duplicate. Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid (TCA) were used to correct for non-enzymatic hydrolysis of substrates. Enzyme activity was expressed as nmol of phosphate (P_i) released min^{-1} mg $protein^{-1}$.

Statistical analysis

Results were analyzed by one-way, two way or three-way analysis of variance (ANOVA) and this is indicated in text of results. Duncan's Multiple Range Test and paired *t* test were applied where appropriate. Differences between groups were considered to be significant when $P < 0.05$.

Results

Effect of organodiselenides on lipid peroxidation induced by iron and sodium nitruprusside

Three-way ANOVA (2 (DPDS/DCDS) × 5 concentrations of selenides × 2 (basal/iron) revealed a significant third-order interaction ($P < 0.0001$). Interaction was significant because, as can be seen in Fig. 1A, DPDS was able to exert significant inhibitory effect on basal and iron induced lipid peroxidation in the low-speed supernatant from brain homogenate. However, DCDS exert a prooxidant like effect on basal and iron induced lipid peroxidation in the brain.

Three-way ANOVA (2 (DPDS/DCDS) × 5 concentrations of selenides × 2 (basal/sodium nitroprusside)) revealed a significant third-order interaction ($P < 0.0001$). Interaction was significant because, as can be seen in Fig. 1B, DCDS elicited a prooxidant effect on the basal production of TBARS by brain low-speed supernatants (S1), but not in the presence of sodium nitroprusside. Sodium nitroprusside caused a marked increase in TBARS production and this effect was not modified by the DCDS. In contrast, DPDS exerted a profound antioxidant effect both on the basal and sodium nitroprusside-stimulated TBARS production.

Effect of organodiselenides on formation of protein carbonyls induced by iron

Figure 2 shows the effect of the two diselenides on the formation of protein carbonyls in the low-speed supernatant from brain homogenate. Three-way ANOVA revealed that iron (II) induced a marked increase on the formation of protein carbonyls which was markedly inhibited by DPDS in a concentration dependent manner. However, the DCDS did not have any protective effect on the formation of protein carbonyls at all the concentrations tested. This was indicated by the significant third order interaction (2 (DPDS/DCDS) × 6 concentrations of selenides × 2 (basal/iron (II); $P < 0.01$)

Glutathione peroxidase mimetic activity of organodiselenides

Three way ANOVA of glutathione peroxidase activity of selenides (2 compounds × 2 conditions: with and without H_2O_2 × 7 sample times) revealed a significant third-order interaction ($P < 0.001$). In line with this, the rate of GSH

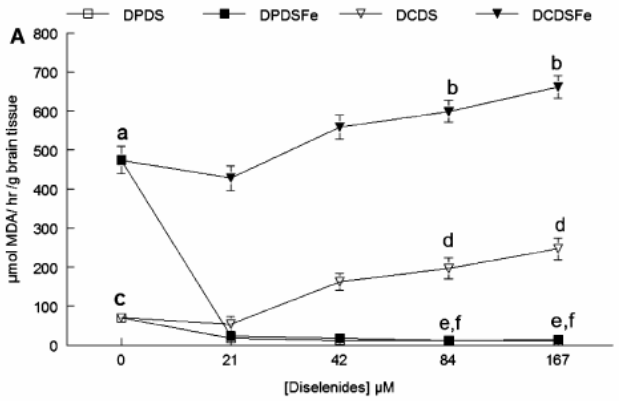


Fig. 1 (A) Effect of different concentrations of DPDS and DCDS on basal and iron (II) (10 μM)—induced TBARS production in the low-speed supernatant from brain homogenate. (**B**) Effect of different concentrations of DPDS and DCDS on basal and SNP (3 μM)—induced TBARS production in the low-speed supernatant from brain homogenate. Data show mean ± SEM values average from four independent experiments performed in quadruplicate in different days. Data are presented as mean ± SEM and post-hoc comparisons were done by Duncańs multiple range test. Different letters indicate a significant difference in relation to the control (no compounds, but with prooxidant) or to basal (no pro-oxidant) at $P < 0.05$

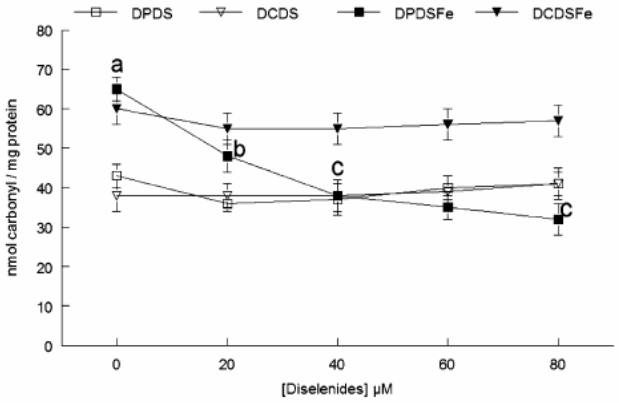


Fig. 2 Effect of DPDS and DCDS on the formation of protein carbonyls in the low-speed supernatant from brain homogenate in the absence and presence of 5 μM Fe^{2+}. Data are presented as mean ± SEM for 5 independent experiments done in duplicate in different days. Three-way ANOVA was followed by Duncańs multiple range test. Different letters indicate a significant difference in relation to the control (no compounds, but with prooxidant) at $P< 0.05$

oxidation in the absence of peroxide was similar for control (no selenide) and for the selenides (Fig. 3A); however, in the presence of hydrogen peroxide, the oxidation of GSH was significantly higher in the presence of 100 μM DPDS during all the sampling period (Fig. 3B). In contrast, 100 μM DCDS increased the oxidation of GSH only at 2.5 and 5 min sampling times (Fig. 3C, with minus without H_2O_2).

We also performed a two-way ANOVA (2 compounds × 7 sample times) using as dependent variables the differences between the oxidation of GSH measured in the presence of 100 μM DPDS and DCDS to that in absence of selenides. These results are depicted in Fig. 3C and the results of statistical analysis revealed a significant selenides × sample times interaction ($P < 0.001$). In fact, there was a significant difference in the rate of GSH oxidation between the two selenides and this increase as a function of time of incubation (Fig. 3C). Thus, DPDS demonstrated thiol peroxidase-like activity higher than that of DCDS and similar results were obtained when 50 and 150 μM of organodiselenides were used (data not shown).

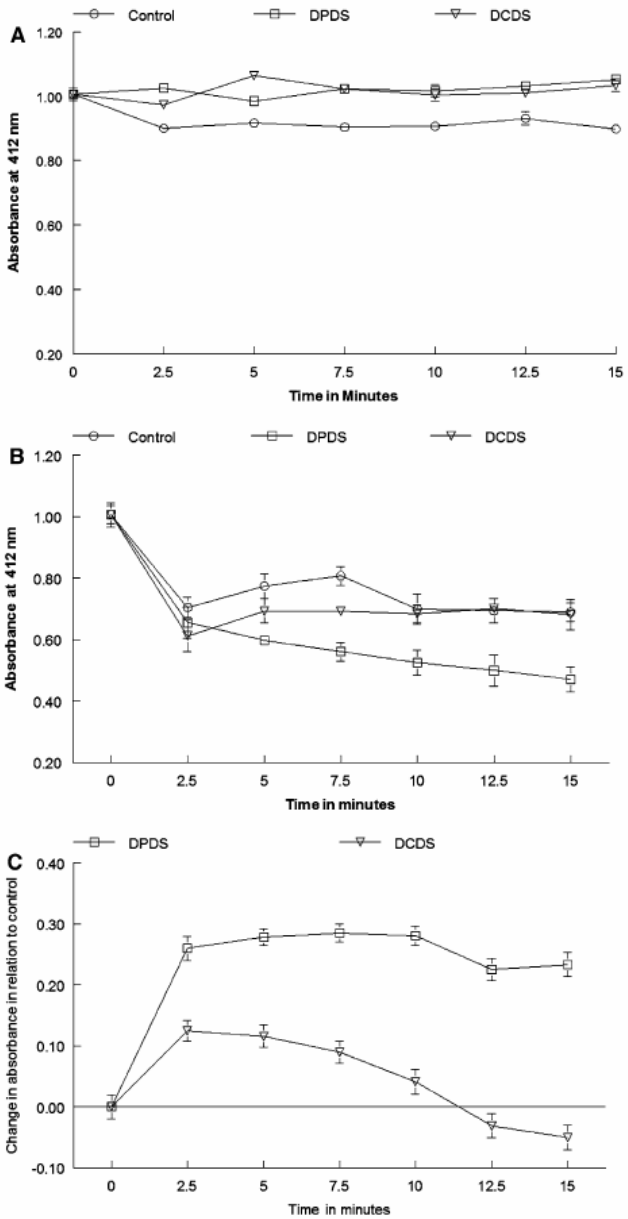


Fig. 3 The peroxidase mimetic activity of DPDS and DCDS (at 100 μM) in the absence (**A**) and in the presence (**B**) of hydrogen peroxide. The peroxidase activity was evaluated at different times range from 0 min to 15 min at 2.5 min intervals. Data are the means of five to seven independent experiments carried out in different days. Data are presented as mean ± SEM and post-hoc comparisons were done by Duncańs multiple range test. (**C**) The difference in the peroxidase mimetic activity of DPDS and DCDS (at 100 μM) in the presence of hydrogen peroxide. The difference in peroxidase activity was calculated at different times range from 0 min to 15 min at 2.5 min intervals. Data are the means of five to seven independent experiments done in different days. Data are expressed as mean ± SEM and post-hoc comparisons were done by Duncańs multiple range test

Effect of organodiselenides on oxidation of mono and dithiols

The rate of cystein oxidation was not modified by organodiselenides (data not shown). Two-way ANOVA (3 compounds: control, DPDS or DCDS × 4 sample times) revealed no significant effects ($P > 0.10$). Similarly, the rate of GSH oxidation was not modified by organodiselenides (data not shown).

The rate of DMPS oxidation was increased by DPDS and DCDS (Fig. 4). Two-way ANOVA (3 compounds: control, DPDS or DCDS × 4 sample times) revealed a significant organoselenides × sample time interaction ($P < 0.01$). In fact, the oxidation of DMPS was increased as a function of time and its oxidation was accelerated by organoselenides (Fig. 4).

The rate of DTT oxidation was increased by DPDS (Fig. 5). Two-way ANOVA (3 compounds: control, DPDS or DCDS × 5 sample times) revealed a significant organoselenides × time interaction ($P < 0.01$). In fact, the oxidation of DTT was increased as a function of time, but only in the presence of DPDS (Fig. 5).

Effect of organodiselenides on the activity of ð-aminolevulinic acid dehydratase

Analysis of cerebral ∂-aminolevulinic acid dehydratase activity revealed that DPDS caused a significant inhibition of enzyme activity. In contrast, DCDS did not significantly inhibit cerebral ∂-aminolevulinic acid dehydratase. These results were confirmed by a significant organoselenides × concentration interaction (Fig. 6, $P < 0.01$).

Effect of organodiselenides on the activity of Na^+/K^+-ATPase

Three-way ANOVA of Na^+/K^+-ATPase activity (6 pre-incubation conditions × 2 organoselenides × 5 concentrations)

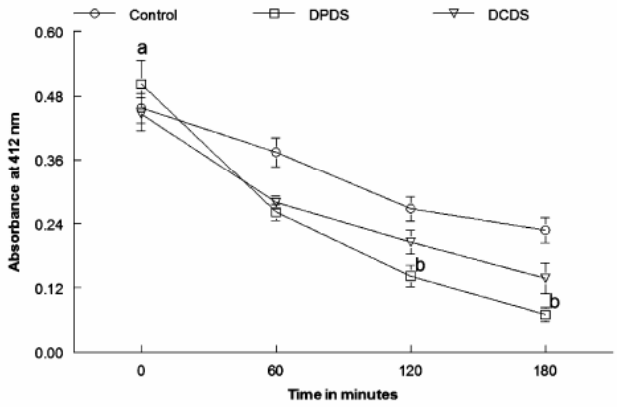


Fig. 4 Effects of DPDS and DCDS on the rate of DMPS (a dithiol) oxidation. The rate of oxidation was evaluated at the indicated times. Data are the means of five to seven independent experiments carried out in different day. Data are expressed as mean ± SEM and post-hoc comparisons were done by Duncańs multiple range test. Different letters indicate a significant difference in relation to the control

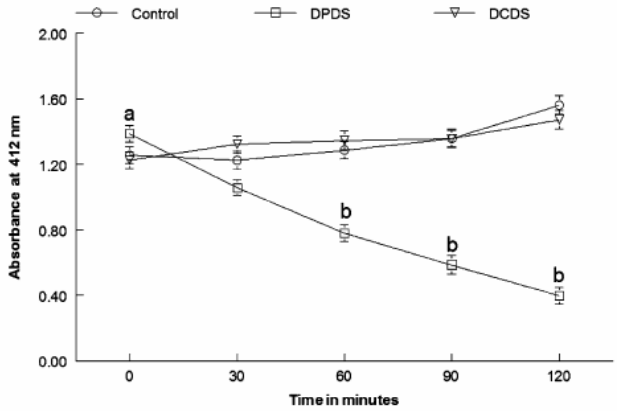


Fig. 5 Effects of DPDS and DCDS on the rate of DTT (a dithiol) oxidation. The rate of oxidation was evaluated at the indicated times. Data are the means of five to seven independent experiments carried out in different day. Data are expressed as mean ± SEM and post-hoc comparisons were done by Duncańs multiple range test. Different letters indicate a significant difference in relation to the control

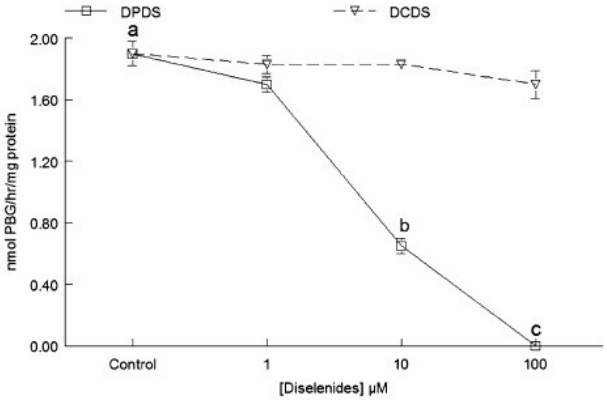


Fig. 6 Effect of DPDS and DCDS on the activity of δ-ALA-D in the low-speed supernatant from brain homogenate. Each point represents the mean ± SEM for four independent assays with different supernatant preparations carried out in different days. Two-way ANOVA was followed by Duncan's multiple range test. Different letters indicate a significant difference in relation to the control (no compounds) at $P < 0.05$

revealed a significant third-order interaction that was a consequence to the fact that the inhibitory effect of diselenides (mainly of DPDS) was markedly affected by the pre-incubation condition (Fig. 7). In order to better explore the influence of pre-incubation condition on the inhibitory effect of selenides, we performed separate statistical analyses (2 organoselenides × 5 concentrations) for each pre-incubation condition. When the enzyme were

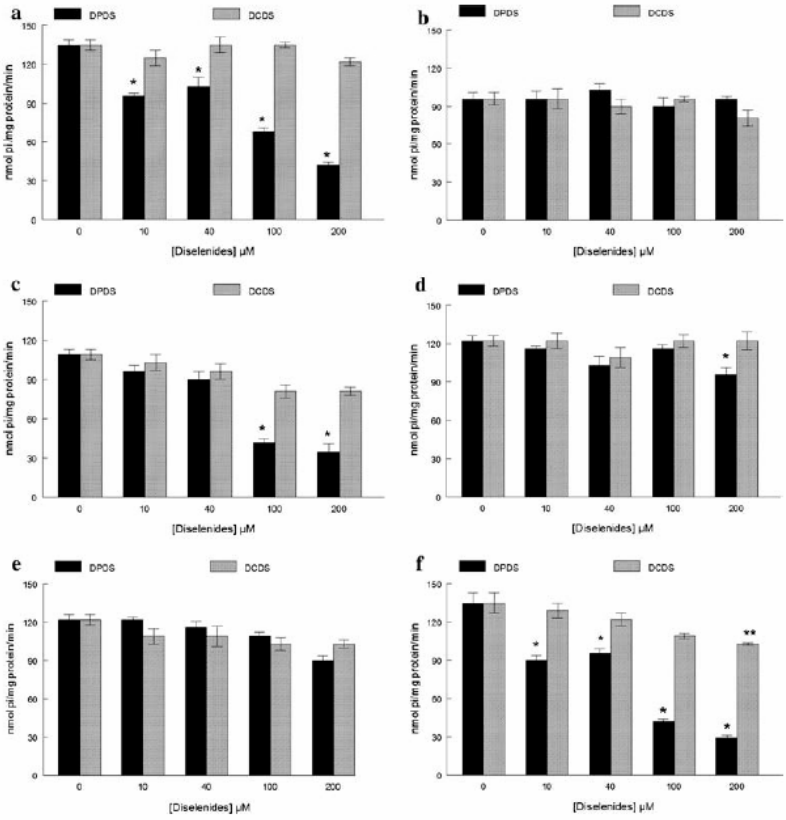


Fig. 7 Influence of pre-incubation conditions on the inhibitory effects of DPDS and DCDS on Cerebral Na^+/K^+-ATPase. (**a**) reaction was started by addition of ATP after 10 min of pre-incubation in the presence of Na^+, K^+ and Mg^{2+}; (**b**) reaction was started by the simultaneous addition of ATP, diorganoselenides and brain enzyme; (**c**) reaction was started by addition of Mg^{2+} after 10 min of pre-incubation in the presence of Na^+, K^+ and ATP; (**d**) reaction was started by addition of Na^+ after 10 min of pre-incubation in the presence of Mg^{2+}, K^+ and ATP; (**e**) reaction was started by addition of K^+ after 10 min of pre-incubation in the presence of Mg^{2+}, Na^+ and ATP; (**f**) reaction was started by addition of Na^+, K^+ and Mg^{2+} after 10 min of pre-incubation in the presence of ATP. Results are expressed as nmol P_i min^{-1} mg $protein^{-1}$. Data represent the mean ± SD of three different experiments carried out in different days and were tested by two-way ANOVA followed by Duncan's test. *Indicates a significant difference from control (no diselenide) at $P < 0.05$

pre-incubated for 10 min in the presence of Na^+, K^+ and Mg^{2+} (pre-incubation without ATP), two-way ANOVA revealed a significant organoselenide type × concentrations interaction ($P < 0.001$). Interaction was significant because DPDS caused a concentration dependent inhibition of ATP hydrolysis, whereas DCDS did not modify the enzyme activity (Fig. 7a). In the absence of pre-incubation (reaction started by the simultaneous addition of ATP and enzyme), two-way ANOVA yielded no significant main or interaction effects (Fig. 7b). Two-way ANOVA of the ATPase activity after pre-incubation of the enzyme for 10 min in a medium containing Na^+, K^+ and ATP (pre-incubation without Mg^{2+}) revealed a significant interaction of organoselenide type × concentrations. In a similar way to that observed when pre-incubation was carried out in the absence of ATP, DPDS caused a concentration dependent inhibition of the ATP hydrolysis and DCDS did not inhibit the enzyme activity (Fig. 7c). Two-way ANOVA of enzyme activity when Na^+ was omitted from the pre-incubation medium yielded a significant diselenide type × concentration interaction ($P<0.05$). This was significant because DPDS caused a significant inhibition of ATP hydrolysis when tested at 200 μM (Fig. 7d). Two-way ANOVA of ATP hydrolysis determined after pre-incubation in the absence of K^+ yielded non-significant main or interaction effects ($P > 0.10$; Fig. 7e). Two-way ANOVA of Na^+, K^+-ATPase determined after 10 min of pre-incubation in the absence of Na^+, K^+ and Mg^{2+} yielded a significant diselenide type × concentration interaction ($P < 0.01$). Interaction was significant because DPDS caused a concentration dependent inhibition of ATP hydrolysis, whereas DCDS caused a significant inhibition of enzyme activity only when tested at 200 μM ($P > 0.05$, Fig. 7f).

Discussion

In our earlier studies we have demonstrated that the substitution of an aromatic moiety of diorganyl chalchogenides could modulate their effects [15, 23]. Here we also verified that the substitution of a cholesteryl moiety in place of phenyl moiety on diselenides changes their antioxidant and thiol peroxidase like-properties. The thiol peroxidase-like activity of diorganyl chalcogenides can explain, at least in part, the in vitro antioxidant properties of these compounds [38–42]. The results indicate that DPDS presented higher thiol peroxidase activity (Fig. 3) and demonstrated better antioxidant potential than DCDS. In addition, DPDS demonstrated higher potential for –SH group oxidation than DCDS (Figs. 4, 5). It is interesting to note that GSH was not oxidized by DPDS or DCDS in the absence of peroxide. This may be a consequence to the fact that GSH has a lower redox potential than dithiols, consequently, GSH has a weak potency to open the Se–Se bound of DPDS, but it can do this when the selenenic acid is formed after reaction with peroxide.

The mechanism by which iron causes deleterious effect is by reacting with superoxide anion ($O_2^{\bullet}$) and hydrogen peroxide (H_2O_2) to produce the hydroxyl radical ($OH^{\bullet}$) via the Fenton reaction [43]. These radicals can also lead to the formation of other reactive oxygen species (ROS) [44]. The overproduction of ROS can directly attack the polyunsaturated fatty acids of the cell membranes and induce lipid peroxidation. However the synthetic DCDS did not protect against iron induced lipid peroxidation (Fig. 1) or protein carbonyls formation (Fig. 3) in the rat brain. In fact, our results (Fig. 1) demonstrate that it acts synergistically to augment lipid peroxidation products in brain. DPDS on the other hand was able to significantly protect the formation of TBARS in the rat brain homogenate subjected to iron (Fig. 1). In addition, attack by ROS upon proteins can damage several amino acid residues, including histidine, tryptophan, cysteine, proline, methionine, arginine and lysine. Oxidative damage to several of these amino acid residues and/or to the peptide backbone of proteins can generate carbonyl products [45]. However, the result presented in Fig. 2 shows that DPDS was able to exert a significant inhibitory effect on the formation of protein carbonyls; on the other hand, the DCDS had no protective effect on formation of carbonyls at all the concentration tested.

Photodegradation process of sodium nitroprusside (SNP) ultimately produces $NO^{\bullet}$, $[(CN)5\text{-}Fe]^{3+}$ and $[(CN)4\text{-}Fe]^{2+}$ species [46–48]. There is a growing number of studies concerning the role of NO, a molecule that is regarded as universal neuronal messenger in the central nervous system, in the pathophysiology of such disorders as Alzheimer's and Parkinson's diseases, stroke, trauma, seizure disorders, etc. [49–52]. NO is a free radical with short half-life (<30 s). Although NO acts independently, it also may cause neuronal damage in cooperation with other reactive oxygen species (ROS) [49, 53]. Thus, the protective effect of DPDS can be related to the ability of its selenol intermediate to react with the potentially toxic NO. Recently, we have observed that DPDS protects endothelial cells from the toxic effect of NO, which strongly indicate that DPDS can scavenge NO.

The iron moiety of SNP may have a free iron coordination site for H_2O_2, which could trigger the generation of highly reactive oxygen species, such as hydroxyl radicals ($OH^{\bullet}$) via the Fenton reaction [43]. Therefore following a short-lasting release of NO in the brain, iron moiety of SNP could cause a long-lasting generation of $OH^{\bullet}$ radicals and oxidant stress/injury similar to that of ferrous citrate iron complexes which may initiate a lipid peroxidation chain reaction and oxidative brain injury [54]. The result

presented in Fig. 1B indicated that while the DPDS exerted an antioxidant effect on in vitro sodium nitroprusside induction of lipid peroxidation in brain homogenate, whereas DCDS had no antioxidant effect. The absence of antioxidant activity of DCDS may be related to its low glutathione-peroxidase like activity. As consequence of this low activity, DCDS is a weaker scavenger of OH* radical. Furthermore, since the thiol-peroxidase activity and the ability of organoselenium compounds to scavenge free radicals will depend in their ability to form a selenol [15], we can suppose that the bulky moiety DCDS hampers the opening of the Se–Se bond and accounts for its low antioxidant activity.

δ-Aminolevulinate dehydratase (δ-ALA-D) is a sulfhydryl containing enzyme that is inhibited by a variety of sulfhydryl reagents [5, 15, 55–58]. This enzyme catalyzes the condensation of two ∂-aminolevulinic acid (ALA) molecules with the formation of porphobilinogen, which is a heme precursor [59]. Consequently, δ-ALA-D inhibition may impair heme biosynthesis [60] and can result in the accumulation of ALA, which may affect the aerobic metabolism and may have some prooxidant activity [61]. DPDS inhibited the activity of the enzyme in a concentration dependent manner and at 100 μM, it exerted a complete inhibition on its activity (Fig. 6). However the novel DCDS exerted only a weak (< 10%) non-significant inhibitory effect on the activity of ALA-D. Earlier studies from our laboratory [15] have shown that the inhibition of δ-ALA-D by selenium compounds involves the oxidation of essential SH groups of the enzyme. In line with this, DPDS oxidized DTT (a dithiol), while DCDS was not effective as oxidant of DTT. Therefore with reference to our earlier observation, the rate of oxidation of DTT could explain the observed inhibition of the thiol containing protein ∂-ALA-D.

The effect of the organodiselenides on another thiol containing protein, Na^+K^+-ATPase was also studied. Our present results (Fig. 7a, c, f) clearly indicate that DPDS inhibit Na^+, K^+-ATPase activity while DCDS inhibited the enzyme only at the highest concentration and only after the pre-incubation was carried out in the absence of Na^+, K^+ and Mg^{2+}. Borges et al. [22] observed that dithiothreitol was able to revert the inhibition caused by DPDS and suggested that the possible mechanism of inhibition of the ATPase enzyme by organodiselenides may be related to their interaction with cysteinyl residues that are important for the enzyme activity. Other reports have demonstrated the importance of thiol groups for Na^+, K^+-ATPase catalysis. In fact, –SH groups of this enzyme is highly susceptible to oxidizing agents [62–64]. In addition, further studies (Fig. 7) with selective exclusion of each of the component of the cations in the incubating medium reveal that the inhibition of Na^+/K^+ ATPase by DPDS may possibly involve the modification of the ATP binding site of the enzyme. In conformity with our earlier observation, we may further conclude that the observed inhibition of Na^+/K^+ ATPase by organodiselenides, possibly involves the modification of the critical –SH group at the ATP binding site of the enzyme. In fact, the exclusion of magnesium from the incubating medium possibly impose a structural deformity on the magnesium dependent ATP, thereby preventing the competitive binding of the ATP to its site in the presence of organodiselenides. This hypothesis is strongly supported by the fact that inclusion of magnesium cation in the incubating medium abolished the observed inhibitory effect by DPDS (Fig. 7b, c, f). This is the first study in our laboratory suggesting the possible interaction of DPDS with the critical –SH at the ATP binding site of the cerebral Na^+ K^+ ATPase.

In conclusion, the results of the present investigation indicate that DPDS can be considered more potent oxidant of thiols than DCDS. However, DPDS also can use thiols to reduce peroxides, which can accounts for its pharmacological properties as potential scavenger of free radicals. Since DCDS presented a weak ability to oxidize thiols and to decompose hydrogen peroxide, we can expect that it will be less effective than DPDS as a potential scavenger of oxygen free radical. In contrast, since the toxicity of diselenides can be related to their ability in oxidize thiol groups we can suppose that DCDS should be less toxic than DPDS.

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3.1.2 Second Manuscript

STUDIES ON THE ANTIOXIDANT EFFECT AND INTERACTION OF DIPHENYL DISELENIDE AND DICHOLESTEROYL DISELENIDE WITH HEPATIC DELTA AMINOLEVULINIC ACID DEHYDRATASE AND ISOFORMS OF LACTATE DEHYDROGENASE

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(Submitted to Toxicology *In Vitro*)

STUDIES ON THE ANTIOXIDANT EFFECT AND INTERACTION OF DIPHENYL DISELENIDE AND DICHOLESTEROYL DISELENIDE WITH HEPATIC DELTA AMINOLEVULINIC ACID DEHYDRATASE AND ISOFORMS OF LACTATE DEHYDROGENASE

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ABSTRACT

Studies on the interaction of dicholesteroyl diselenide (DCDS) and diphenyl diselenide (DPDS) with hepatic ð-aminolevulinic acid dehydratase (ALA-D) and different isoforms of lactate dehydrogenase (LDH) from different tissues were investigated. In addition, their antioxidant effects were tested *in vitro* by measuring the ability of the compounds to inhibit the formation of hepatic thiobarbituric acid reactive species (TBARS) induced by both iron (II) and sodium nitroprusside (SNP). The results show that while DPDS markedly inhibited the formation of TBARS induced by both iron (II) and SNP, DCDS did not. Also, the activities of hepatic delta aminolevulinic acid dehydratase (ALA-D) and different isoforms of lactate dehydrogenase (LDH) were significantly inhibited by both DPDS and DCDS. Moreover, we further observed that the *in vitro* inhibition of different isoforms of lactate dehydrogenase by DCDS and DPDS likely involves the modification of the groups at the NAD^+ binding site of the enzyme. Since organoselenides interacts with thiol groups on proteins, we conclude that the inhibition of different isoforms of lactate dehydrogenase by DPDS and DCDS possibly involves the modification of the thiol groups at the NAD^+ binding site of the enzyme.

Keywords: Organoselenium compounds; ð-ALA-D; isoforms of lactate dehydrogenase; antioxidants; TBARS.

INTRODUCTION

Physiologically, selenium is an essential element that participates in the antioxidative defense systems (Mugesh *et al*., 2001; Nogueira *et al*. 2004). Essentially, there are claims that the organoselenium compounds such as Ebselen and diorganyl diselenide exhibit both glutathione peroxidase (GSH-Px)-mimetic activity as well as thioredoxin peroxidase mimetic activity (Arteel and Sies, 2001; Zhao and Holmgren, 2002) and these properties of organoselenium compounds may be related in parts to their observed *in vitro* antioxidative activity making them promising candidates as medicaments in the management of a number of degenerative diseases in which oxidative stress have been implicated in their aetiology (Nogueira *et al*., 2001, 2004). Hence, the synthesis and evaluation of pharmacological potency of selenium and its organo-compounds have continued to attract the attention of researcher for some decades (Nogueira, *et al*., 2004). Ebselen, the first reported synthetic organoselenium compound, is a complex molecule and consequently expensive to synthesize. Hence synthesis and biological testing of other simpler and inexpensive forms of organoselenium compounds such as the diorganyl diselenide: diphenyl diselenides (DPDS), akyl and aryl diselenides with promising pharmacological potency have been given attention in recent times (Mugesh, *et al*., 2001). In fact, we recently described the possible pharmacological potency of a novel diorganyl diselenide derived from two cholesterol steroidal molecules, dicholesteroyl diselenide (DCDS) which was recently synthesized in our laboratory (Kade *et al*., 2008).

Although selenium compounds generally hold promise as potent antioxidant drugs, they are generally poisonous to mammalian systems (Nogueira *et al*., 2003), and in fact, despite considerable efforts at understanding precise mechanism involved in toxicity elicited by selenium compounds (Nogueira *et. al*., 2004; Mugesh *et al*., 2001), chemistry of selenium

poisoning is still an enigma: the toxic symptoms are complex and the mechanism of the poisoning is still obscure. Some investigators believe that selenium compounds exert their toxic effect through interference with certain enzyme systems in the living organism, particularly through mercaptide formation with important sulphydryl enzymes with physiological importance in biological systems (Nogueira *et al*., 2004). Two of these sulphydryl enzymes are δ-Aminolevulinate dehydratase (δ-ALA-D) and lactate dehydrogenase.

δ-Aminolevulinate dehydratase (δ-ALA-D) is an important sulfhydryl containing enzyme (Rocha, *et al.,* 1995) that catalyses the condensation of two ð-aminolevulinic acid (ð-ALA), yielding porphobilinogen (PBG) which is a heme precursor (Jaffe, 1995). Inhibition of ALA-D can block this pathway causing accumulation of heme intermediates, including its substrate, δ-ALA, which has been reported to posses some pro-oxidant activity under physiological conditions (Bechara, 1996). Earlier studies in our laboratory (Barbosa *et al*., 1998) have shown that the inhibition of δ-ALA-D by selenium compounds involves the oxidation of essential SH groups of the enzyme, since thiol protecting groups such as DTT and reduced glutathione (GSH) can reverse this inhibition. In addition, we also observed that the mechanism underlying the inhibitory effect of these compounds on δ-ALA-D is related to the oxidation of essential cysteinyl residues located at the active site of the enzyme.

On the other hand, lactate dehydrogenase (LDH), another thiol containing enzyme reversibly converts pyruvate and NADH into lactate and NAD^+. Generally, the isoenzymes of LDH are tetramers formed from two types of monomers (Koslowski *et al*., 2002). The two isoforms are labeled H (heart) and M (muscle), (formerly referred to as B and A respectively) and their ratio varies between cell types. The LDH isoform ratio has long

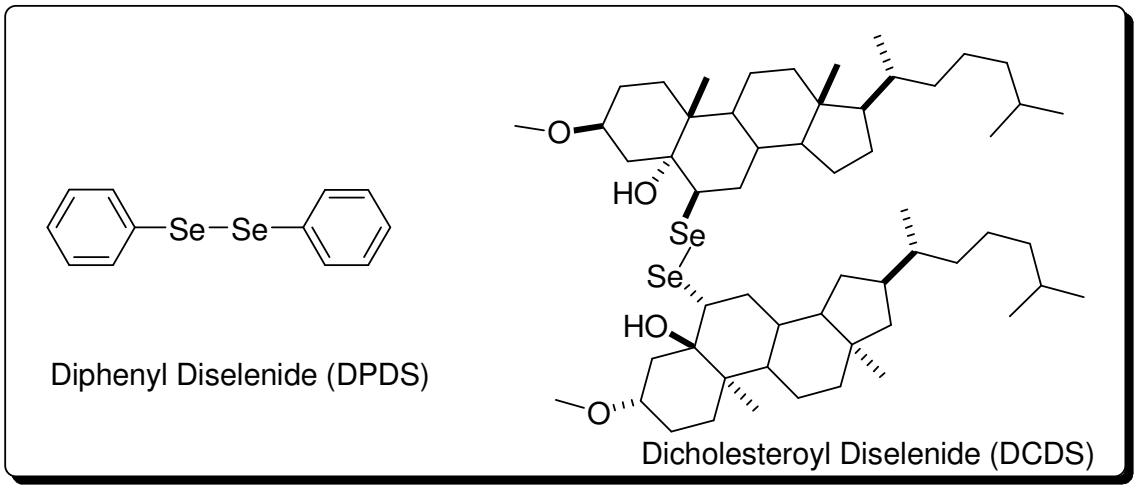
been proposed to indicate the metabolic state of cells: it is believed that the M isoform favors lactate production while the H isoform favors pyruvate production (Stambaugh and Post, 1966). Therefore, LDH isoform ratio can serve as an indicator of the relative flux through aerobic/anaerobic gycolytic pathways. The enzyme contains two main domains: the coenzyme (nicotinamide adenine dinucleotide, reduced [NADH] or nicotinamide adenine dinucleotide [NAD^+] binding domain, which is highly conserved in the dehydrogenases (the "Rossmann fold"), and the substrate binding domain (Kutzenko *et al*., 1998). All isoforms of lactate dehydrogenases contain zinc as a functional component of the active site (Vallee and Wacker, 1956) and can be inhibited by sulfhydryl-binding reagents, such as p-chloromercuribenzoato (Neilands, 1954).

In our recent report (Kade *et al.,* 2008), we observed that DCDS in comparison with DPDS have a weak glutathione peroxidase (GSH-Px)-mimetic activity and also a weak ability to oxidize both mono (glutathione and cysteine) and dithiols (DMPS, DMSA and DTT), explaining the reasons for the observed weak antioxidant effect and inhibition of thiol containing enzymes (cerebral ALA-D and Na^+/K^+-ATPase) by DCDS. However, since we earlier observed that the non-selenium moiety of the organochalcogens can have a profound effect on their antioxidant activity and also exert differential effects in their reactivity towards –SH groups from low-molecular weight molecules and proteins (Kade *et al.,* 2008), the present study therefore sought to compare the antioxidant potential as well as the interaction of DPDS and DCDS another two thiol containing proteins, namely, δ-ALA-D and different isoforms of lactate dehydrogenase.

2.0 MATERIALS AND METHODS

2.1 Chemicals

DPDS and DCDS (Scheme 1) were synthesized according to literature methods (Paulmier, 1986). These drugs were dissolved in 99% Ethanol. Analysis of the ^{1}HNMR and ^{13}CNMR spectra showed that all the compounds obtained presented analytical and spectroscopic data in full agreement with their assigned structures. The purity of the compounds were assessed by high resonance mass spectroscopy (HRMS) and was higher that 99.9%. All other chemicals used were of analytical grade and obtained from Sigma Aldrich, FLUKA, BDH and other standard commercial suppliers.



Scheme 1: Structure of diphenyl diselenide and dicholesteroyl diselenide

2.2 Animals

Male adult Wistar rats (200–250 g) from our own breeding colony were used. Animals were kept in separate animal rooms, on a 12-h light: 12-h dark cycle, at a room temperature of 22-24 °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 RS, Brazil.

2.3 Preparation of tissue homogenate for thiobarbituric acid reactive species (TBARS) assay

Rats were decapitated under mild ether anesthesia and the liver tissues was rapidly dissected, placed on ice and weighed. Tissues were immediately homogenized in cold 10mM Tris–HCl, pH 7.5 (1/10, w/v) with 10 up-and-down strokes at approximately 1200 rev/min in a Teflon-glass homogenizer. The homogenate was centrifuged for 10 min at 4000×*g* to yield a pellet that was discarded and a low-speed supernatant (S1). An aliquot of 100 µl of S1 was incubated for 1 h at 37 °C in the presence of both organodiselenides (final concentrations range of 21-167µM previously dissolved in absolute ethanol), with and without the prooxidants; iron (final concentration(10µM) and sodium nitroprusside (final concentration 3 µM). This was then used for lipid peroxidation determination. One rat was used per experiment. Production of TBARS were determined as described by Ohkawa *et al*. (1979) excepting that the buffer of color reaction have a pH of 3.4. The color reaction was developed by adding 1.52% (final concentration) sodium dodecyl sulphate (SDS) to S1, followed by sequential addition of 500 µl acetic acid/HCl (pH 3.4) and 0.25% (final concentration) thiobarbituric acid (TBA). This mixture was incubated at 95 °C for 1 h. TBARS produced were measured at 532 nm and the absorbance was compared to that of a standard curve obtained using malondialdehyde (MDA).

2.4 ð-Aminolevulinic acid dehydratase (ð -ALA-D) activity

Hepatic ð -ALA-D activity was assayed according to the method of Sassa (1982) by measuring the rate of product [porphobilinogen (PBG)] formation except that 100 mM potassium phosphate buffer and 2.5 mM ð-ALA were used. Rat's liver were homogenized in Tris HCl in the proportion 1:10 (w/v) and centrifuged at 4000g for 15 min to yield a low

speed supernatant S1. An aliquot of 100 µl of S1 was incubated for 1 h at 37^0C. Reaction was linear in relation to protein and time of incubation. The reaction product was determined using modified Erhlich's reagent at 555 nm.

2.5 Effect of diselenides on lactate dehydrogenase (LDH) activity *in vitro*

Rats were decapitated under mild ether anesthesia and tissues (liver, kidney and heart) were rapidly dissected, placed on ice and weighed. Tissues were immediately homogenized in cold 10mM Tris–HCl, pH 7.4 (1/10, w/v for liver; 1/5 w/v for both kidney and heart) with 10 up-and-down strokes at approximately 1200 rev/min in a Teflon-glass homogenizer. The homogenate was centrifuged for 10 min at 12000×*g* to yield a pellet that was discarded and a high-speed supernatant (S2). LDH activity in the supernatant (S2) was monitored spectrophotometrically by the rate of increase in absorbance at 340 nm at 35°C resulting from formation of NADH. The assay medium contained 21 mM of glycine-KOH buffer (pH 10), 0.25 mM of NAD^+, diselenides (1-10 µM) and 30µL of liver (15 to 20µg of protein) or cardiac (15 to 20µg of protein) or kidney (12 to 18 µg of protein) or purified lactate dehydrogenase (from rabbit heart) (0.0015µg of protein), The mixture was preincubated for 3 min, and the reaction was started by adding neutralized lactic acid (pH 6.8) to provide a final concentration of 50 mM.

In the inhibition studies, the activity of LDH was assayed by initiating the reaction with simultaneous addition of both lactate and NAD^+, or by selective exclusion of either NAD^+ or lactate in the preincubating medium. In all cases of assay, the reaction was linear for up to 2 min. Also, incubation times and protein concentration were chosen to ensure the linearity of the reactions. All samples were run in triplicate. Protein was determined by the method of Lowry *et al*., (1951). Values are expressed as change in absorbance/mg protein/minute.

2.6 Statistical analysis

Results were analyzed by one-way, two-way or three-way analysis of variance (ANOVA) and this is indicated in text of results. Duncan's Multiple Range Test and paired t test were applied where appropriate. Differences between groups were considered to be significant when $P < 0.05$.

3.0 RESULTS

3.1 Effect of organodiselenides on hepatic lipid peroxidation induced by iron

Three-way ANOVA revealed a significant third-order interaction ($p<0.0001$). Interaction was significant because, as can be seen in figure 1, DPDS was able to exert significant inhibitory effect on iron induced lipid peroxidation in the liver homogenate. Whereas, there was an apparent increase in TBARS level in both the basal and iron stimulated hepatic TBARS production in the presence of DCDS. In addition, for all concentrations of diselenides, TBARS levels in the presence of DPDS and DCDS were significantly different ($P <0.0001$) when iron (II) was used as the prooxidant.

3.2 Effect of organodiselenides on lipid peroxidation induced by sodium nitroprusside

Again, three-way ANOVA (2 (DPDS/DCDS) X 5 concentrations of selenides x 2 (basal/sodium nitroprusside)) revealed a significant third-order interaction ($p<0.0001$). Interaction was significant because, as can be seen in figure 2, DCDS elicited a concentration dependent increase on the basal production of TBARS by liver homogenates but not in the presence of sodium nitroprusside. This increase was significant at 167μM ($P < 0.005$). In contrast, although DPDS did not exert a significant change in TBARS level in

basal, it diminished sodium nitroprusside-stimulated production of TBARS at all concentration ($P < 0.0001$) (Figure 2). Comparing the antioxidant activities of both diselenides (DPDS and DCDS) on SNP-stimulated TBARS production, we observed that there were significant difference at 21 μM ($P < 0.001$), 42, 84 and 167 μM ($P < 0.0001$).

3.3 Effect of organodiselenides on the activity of ð-aminolevulinic acid dehydratase (ð-ALA-D)

Two-way ANOVA (2 organoselenides x 4 concentrations) of hepatic ð-aminolevulinic acid dehydratase activity revealed a significant organoselenides x concentration interaction. DPDS caused a marked concentration-dependent inhibition of ð-ALA-D; however, DCDS caused a concentration dependent inhibition of the enzyme activity which was only significant ($P < 0.0001$) at 100 μM. (Figure 3).

3.4 Effect of organodiselenides on the activity of isoforms of lactate dehydrogensase (LDH)

In order to better explore the influence of pre-incubation conditions on the inhibitory effect of selenides, we performed separate statistical analyses (2 organoselenides x 5 concentrations) for each pre-incubation condition on the isoforms of LDH from the different tissues. Generally comparisons are made in relation to the control values.

3.4.1. Effect of diselenides on renal LDH

When the partially purified renal LDH were pre-incubated for 10 min in the presence of NAD^+ (pre-incubation without lactate) two-way ANOVA shows that the main effect of diselenides was not significant ($F(1, 24) = 0.279$), suggesting that the inhibitory effect of the two diselenides on LDH was similar and in fact DCDS and DPDS generally

diminished the activity of the renal isoforms of LDH which was only significant at 8 and 10µM for DCDS and DPDS respectively. In contrast, when pre-incubation was carried out without NAD^+, the main effect diselenides as well as organoselenide type x concentrations interaction was significant and the results obtained is similar to that obtained when pre-incubation was carried out without NAD^+ and lactate. In fact, we could observe from Figure 4 (B and D), that DCDS markedly inhibited the activity of the enzyme at 2, 4 and 8 µM, while DPDS significantly inhibited the activity of the renal enzyme at all concentration tested. When both lactate and NAD^+ were added simultaneously with diselenides (without preincubation), analysis of data shows that the main effect of diselenide was non-significant ($F(1,24) = 0.98$) because there was no inhibition caused by either DCDS or DPDS.

3.4.2. Effect of diselenides on hepatic LDH

Two-way ANOVA of the results obtained when both DPDS and DCDS were preincubated with the hepatic LDH in the absence of lactate shows that the main effects of both diselenides were not significant ($F(1, 24) = 0.038$). However, two-way ANOVA of data obtained when pre-incubation was carried out without NAD^+ (or both NAD^+ and lactate) yielded a significant main effect for the diselenides. In fact Figure 5 (B and D) shows that the inhibitory effect of DCDS was markedly pronounced at 2, 4, 8 and 10µM, while the inhibitory effect of DPDS was markedly different at 10 µM. Similar to renal LDH, the activity of the hepatic LDH was not inhibited when there was no preincubation. Interestingly, analysis of data gives a non-significant organoselenide type x concentrations interaction ($F(5, 24) = 1.66$).

3.4.3. Effect of diselenides on cardiac LDH

Comparison of data obtained when possible inhibition of the two diselenides (DPDS and DCDS) on cardiac LDH was carried out, apparently shows that both diselenides strongly inhibited LDH activity when NAD^+ (Figure 6B and D) was omitted from the preincubating medium than when lactate (Figure 6A) was absent from the preincubating medium. Two ways ANOVA shows that DCDS significantly inhibited the enzyme activity at 1, 2, 4, and 8 μM, while DPDS markedly inhibited the enzyme activity at 8 and 10 μM. Interestingly, in contrast to both the results obtained for renal and hepatic studies, we observed that without preincubation, (Figure 6D), DCDS (at 4 μM) and DPDS (at 1 μM) significantly inhibited the activity of cardiac lactate dehydrogenase.

3.4.4. Effect of diselenides on purified LDH

Two-ways analysis of variance reveal that the main effect of diselenides ($F(1, 24) = 3.24$) and the interaction of diselenides and concentration of diselenides (($F(1, 24) = 3.24$) on the inhibition of purified LDH in the presence of NAD^+ (pre-incubation without lactate) were not significant. Both diselenides markedly inhibited the enzyme activity. Also, two-way ANOVA of data obtained when pre-incubation was carried out without NAD^+ ($F(1,24) = 0.087$) (or both NAD^+ and lactate ($F(1,24) = 1.06$)) revealed that the main effect of diselenides was not significant. DCDS markedly inhibited the enzyme activity at 1, 2, 4, and 8 μM while DPDS significantly inhibited the enzyme activity at all concentrations tested. Generally when diselenides and the enzyme substrates were added simultaneously (without preincubation), the inhibitory effect of the diselenides were significantly abolished (Figure 7).

DISCUSSION

The present data show that antioxidant potency of DPDS was higher than that of DCDS at all concentrations and under different prooxidant namely iron (II) and SNP (Figures 1 and 2). In fact while DPDS exerted a significant antioxidant effect under iron (II), DCDS exhibited a prooxidant effect. Since iron (II) has been postulated to induce oxidative stress via the Fenton reaction leading to the formation of reactive oxygen species (Harris *et al*., 1992), it follows that DPDS rather than DCDS interact with the Fenton processes. In contrast to potentiating an increase level of TBARS as observed under iron (II) induced TBARS, DCDS significantly diminished the effect of SNP induced TBARS.

SNP has been shown to undergo photodegradation ultimately producing NO^{*}, $[(CN)5\text{-}Fe]^{3+}$ and $[(CN)4\text{-}Fe]^{2+}$ species (Arnold *et al*., 1984). Likewise, it has been observed that the iron moiety of SNP may have a free iron coordination site for H_2O_2, which could trigger the generation of highly reactive oxygen species, such as hydroxyl radicals (OH^{*}) via the Fenton reaction (Graf *et al*., 1984). Therefore following a short-lasting release of NO, iron moiety of SNP could cause a long-lasting generation of OH^{*} radicals and oxidant stress/injury similar to that of ferrous citrate iron complexes which may initiate a lipid peroxidation chain reaction and oxidative injury (Mohanakumar, *et al*., 1994). In addition, nitric oxide has been proposed to act as a pro-oxidant at high concentrations, or when it reacts with superoxide, forming the highly reactive peroxynitrite ($ONOO^{-}$) (Radi *et al*., 2001). Therefore we speculate that both DPDS and DCDS may possibly act to diminish TBARS formation in the presence of SNP by interacting and, consequently scavenging NO or its derivatives via interaction with their respective selenols in a manner similar to the mechanism earlier observed for Ebselen (Mugesh, *et al*., 2001; Nogueira, *et al.,* 2004). Also since DPDS can interact as an antioxidant under Fenton reaction (Figure 1), we further speculate that DPDS may further interact with the cyanide moiety or even the release of

iron from the ferrocyanide moiety of SNP thereby exerting a more profound antioxidant effect than DCDS.

ð-ALA-D is extremely sensitive to the presence of pro-oxidants elements, which can oxidize its –SH groups during oxidative stress (Fernandez-Cuartero *et al*., 1999). Of particular importance, this enzyme is inhibited by both selenium and tellurium compounds that oxidize essential -SH groups of ð-ALA-D (Maciel *et al*., 2000). The extent of inhibition of SH groups of enzymes by organodiselenides have earlier been observed (Barbosa *et al.*, 1998) to positively correlate with their ability to oxidize thiol groups *in vitro.* In conformity with our earlier observation, DPDS completely inhibited the activity of hepatic ð-ALA-D at 100µM; however, DCDS exhibited a concentration dependent inhibition of the activity of the enzyme which was significant at 100 µM. In a related study and as an extension of this work, we earlier demonstrated that DPDS have a higher reactivity with both mono and dithiols groups than DCDS (Kade *et al*., 2007) and consequently, this could explain the observed higher inhibition of ð-ALA-D by DPDS relative to DCDS.

Figures 4-7 show the inhibition of LDH by the two different diselenides. We observed that both DPDS and DCDS were able to exert a significant inhibitory effect on the activity of the different isoforms of the enzymes in the concentration range tested (1-10µM). This inhibitory effect was pronounced irrespective of the diselenides. All isoforms of LDH contains sulfhydryl group that are critical to their activity. There are strong points of evidence indicating that depending on pH some of these critical sulfhydryl groups can undergo gradual oxidation with concomitant inactivation which can be prevented by dithiothreitol (Olson and Massey, 1980). While the reactivity of DCDS with thiols is weak (see Kade *et al*., 2008), its ability to inhibit LDH is comparable with DPDS. In fact, another interesting observation from our present data is that the inhibition of LDH by both DPDS

and DCDS may possibly involve the modification of the thiol group at the NAD^+ binding site. This is consequent from the fact that when NAD^+ is present simultaneously with the diselenides (Figures 1C; 2C; 3C and 4C) or in the preincubating medium with the diselenides (Figures 1A; 2A; 3A and 4A), the inhibitory effect of LDH is abolished or at least is significantly reduced. However, in a medium lacking NAD^+ in the preincubating medium or where both lactate and NAD^+ are excluded from the preincubating medium and added simultaneously after 10 minutes preincubation (Figures 1B; 1D; 2B; 2D; 3B; 3D; 4B and 4D), the diselenides significantly inhibited LDH activities irrespective of the isoforms. However, it could be observed that the activity of the cardiac LDH (Figures 6 and 7) was profoundly inhibited even when NAD^+ was present in the incubating medium. Earlier observations (Boyer, 1975; Voet and Voet, 2004) have shown that cardiac isoforms of LDH rather than hepatic or renal favours the production of pyruvate from lactate. Therefore, we speculate also that both diselenides, in addition to interacting with groups at the NAD^+ binding site, may also interacting with the lactate binding site of the cardiac isoforms.

Furthermore, it is noteworthy that the interaction of DPDS and DCDS with ALA-D and LDH varied and this can be due to their differential reactivity with thiols of the enzyme (Kade *et al*., 2008). But more interestingly, the inhibitory effect of DCDS on LDH (in the absence of NAD^+) may indicate that the non-selenium part is important to reach close to the -SH that is exposed only (or mainly) in the absence of NAD^+, perhaps because NAD^+ block-protects the –SH groups or because it gives the enzyme a conformation more resistant to thiol attacking agents. Also speculatively, it is possible that the volume of the non-selenium group hinders the entry of DCDS on the active site of ALA-D. This may also explain in part, the reason for the observed non significant inhibition of cerebral Na^+/K^+-ATPase by DCDS in our recent report (Kade, *et al*., 2008). In conclusion, the reactivity of organodiselenides with thiols of low and high molecular proteins is complex and depended

on the protein in question. It seems that the simpler diselenides seems to react more promptly with dithiols of low molecular weight and react with some proteins (in the present study ALA-D) more readily than a more voluminous compound (DCDS). In contrast, for the case of LDH, the non selenium part of the DCDS apparently gave a more reactivity to the compound.

Acknowledgement

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Nogueira, C.W., Meotti, F.C., Curte, E.N., Pilissa˜ o, C., Zeni, G.Z., Rocha, J.B.T. (2003). Investigations into the potential neurotoxicity induced by diselenides in mice and rats. *Toxicology* 183, 29–37.

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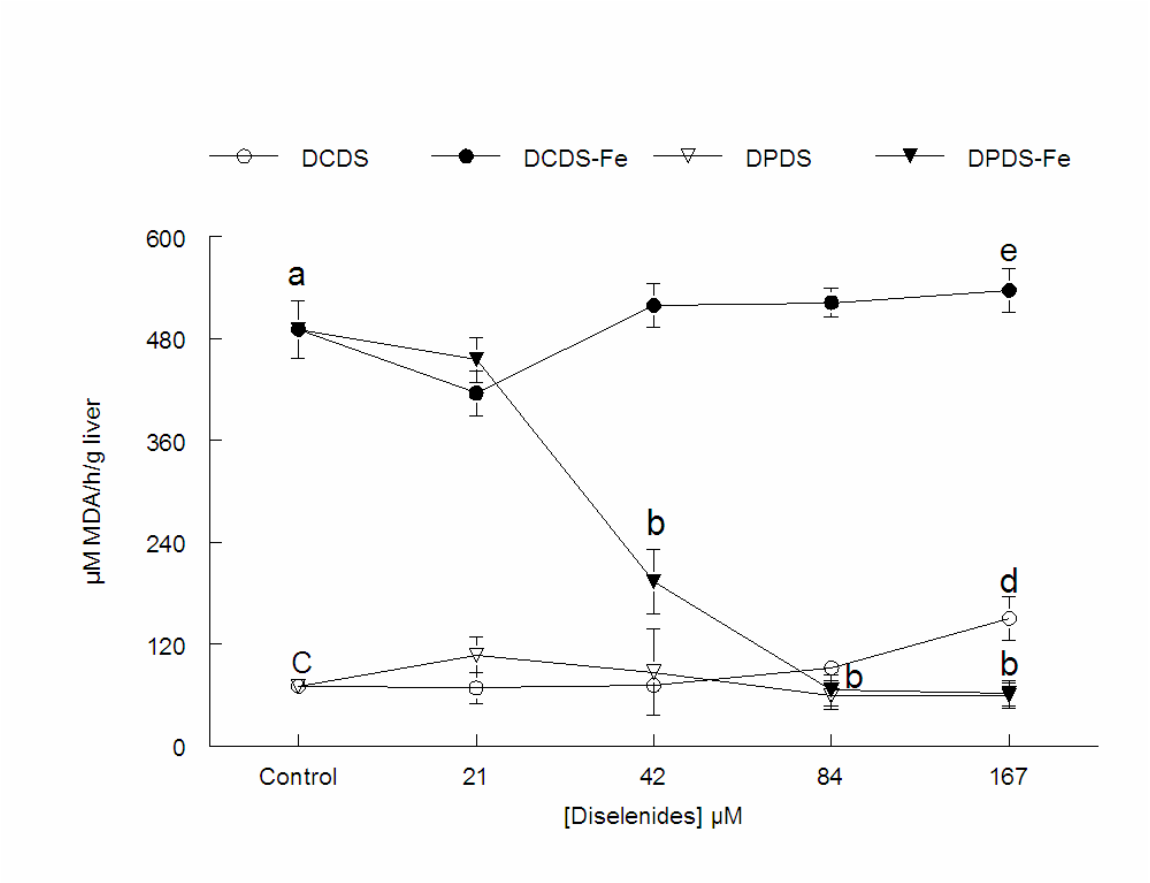


Fig 1. Effect of different concentrations of DPDS and DCDS on basal and iron(II) (10 µM) – induced TBARS production in liver homogenates. Data shows mean±SEM values average from four independent experiments performed in quadruplicate in different days. Letters b and e show marked difference in relation to control a whereas letter d shows marked difference in relation to control c

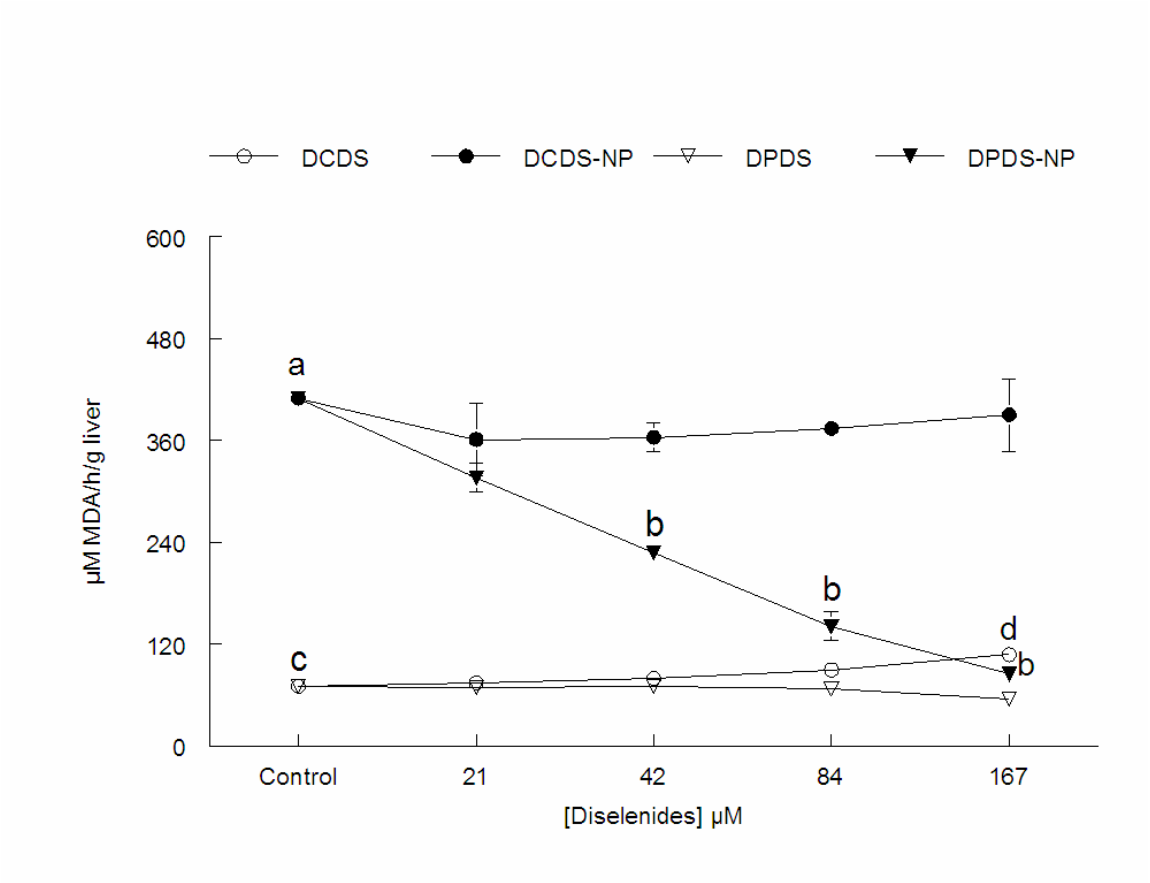


Fig 2. Effect of different concentrations of DPDS and DCDS on basal and SNP (3 µM) – induced TBARS production in liver homogenates. Data shows mean±SEM values average from four independent experiments performed in quadruplicate in different days.

Letter b show marked difference in relation to control a whereas letter d shows marked difference in relation to control c

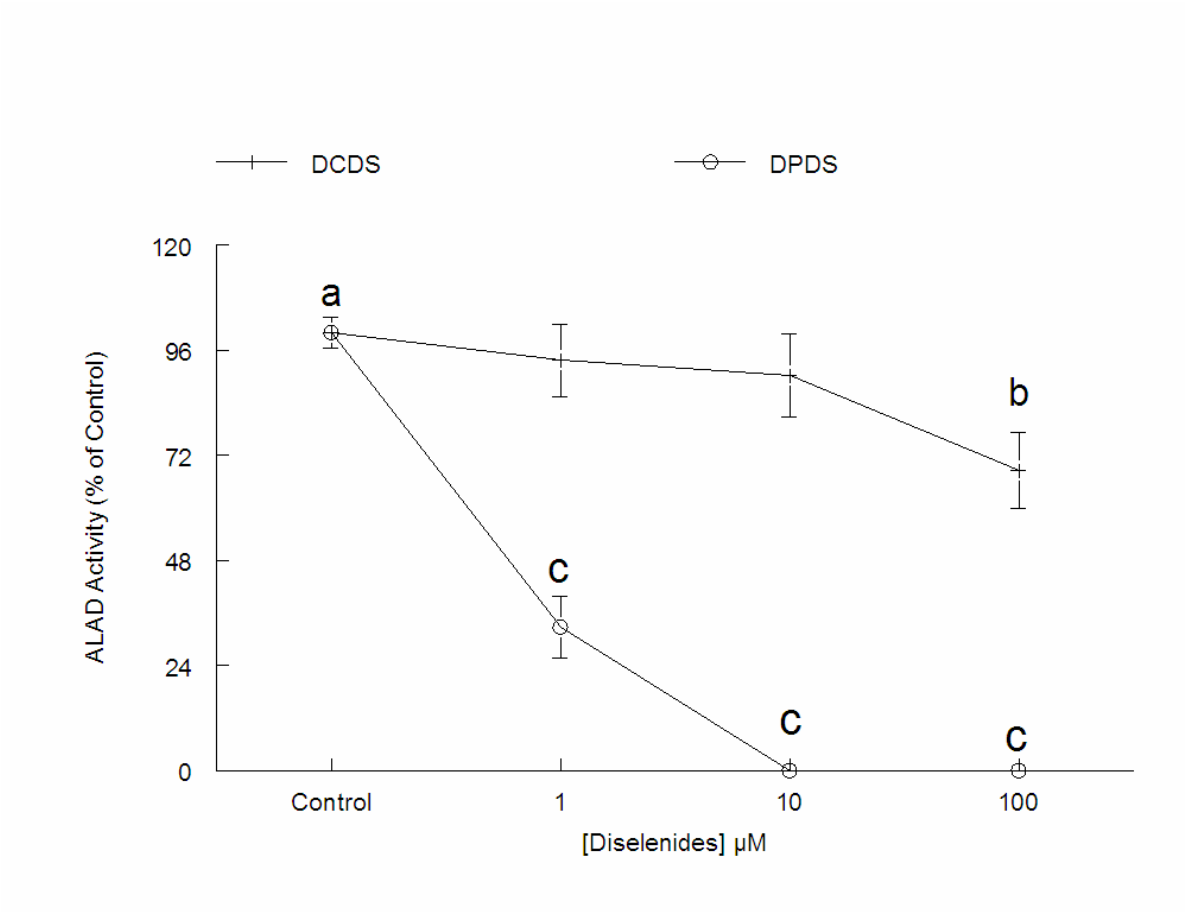
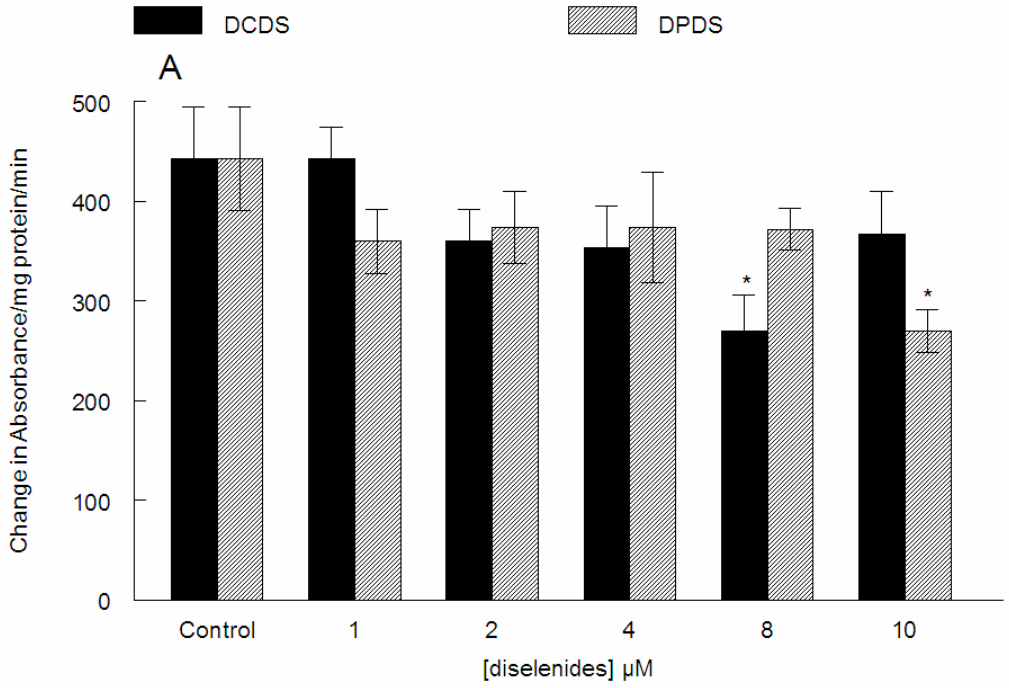


Fig.3: Effect of DPDS and DCDS on the activity of delta-ALA-D from rat liver. Each point represents the mean±SEM for four independent assays with different supernatant preparations carried out in different days. Two-way ANOVA was followed by Duncan´s multiple range test.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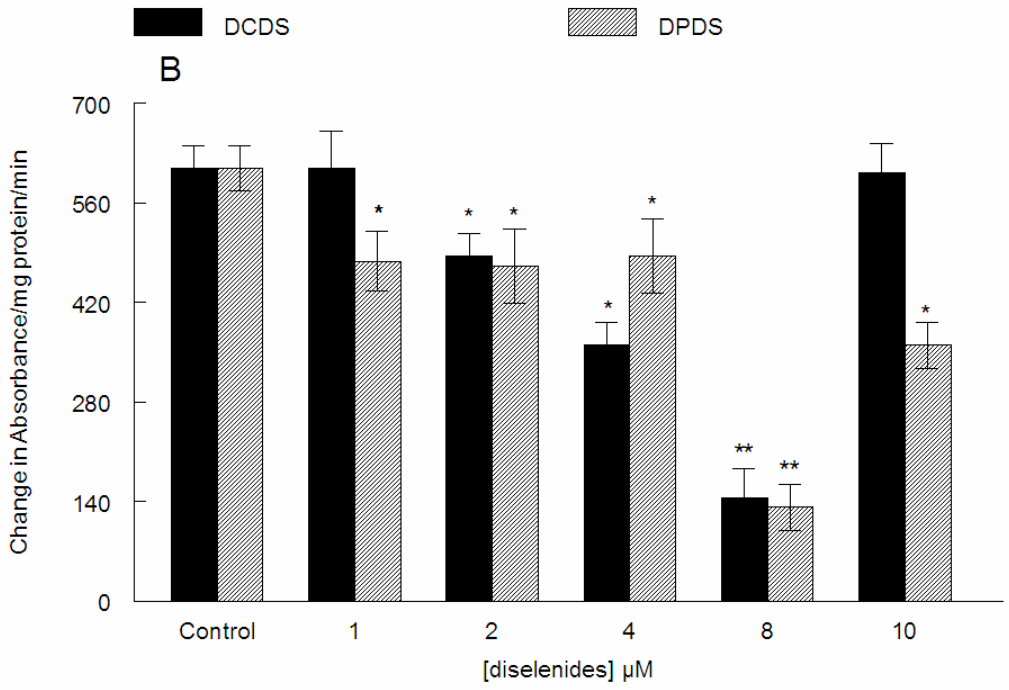
DPDS
A
500
400
300
200
100
0
Change in Absorbance/mg protein/min
Control
1
2
4
8
10
[diselenides] µM
*
*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DPDS
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Change in Absorbance/mg protein/min
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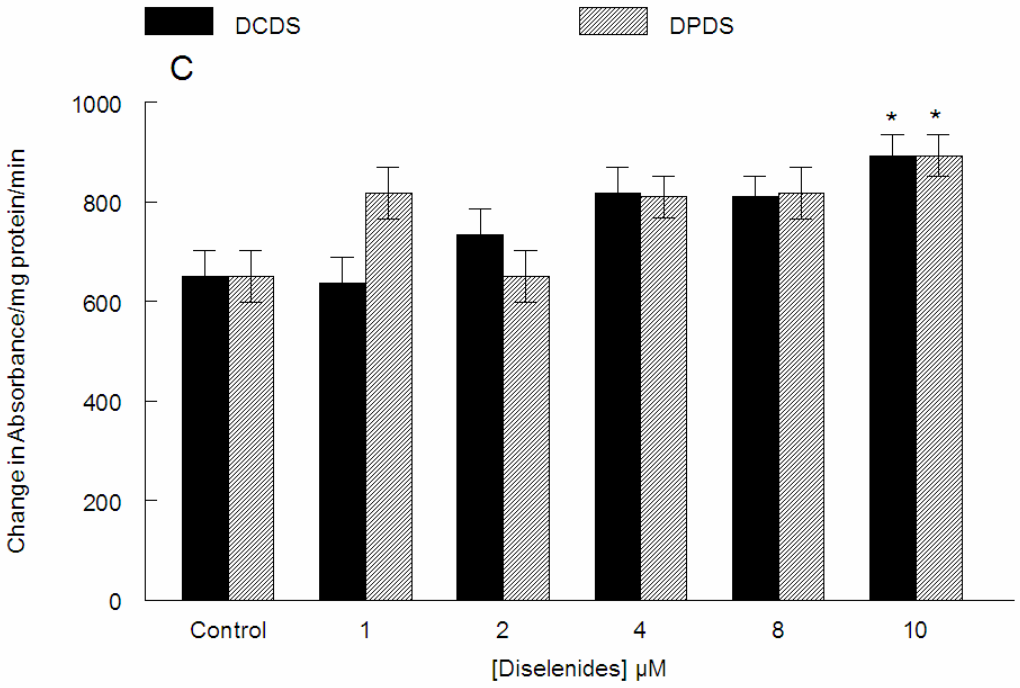
4
8
10
[diselenides] µM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DPDS
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Change in Absorbance/mg protein/min
Control
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[Diselenides] µM
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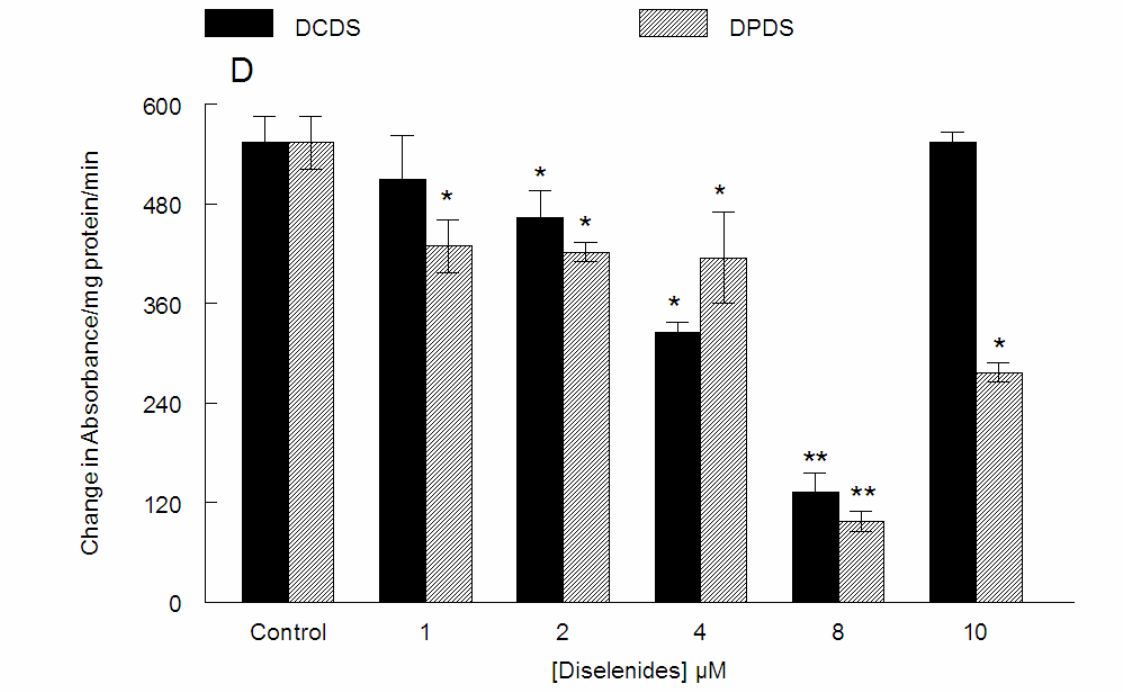
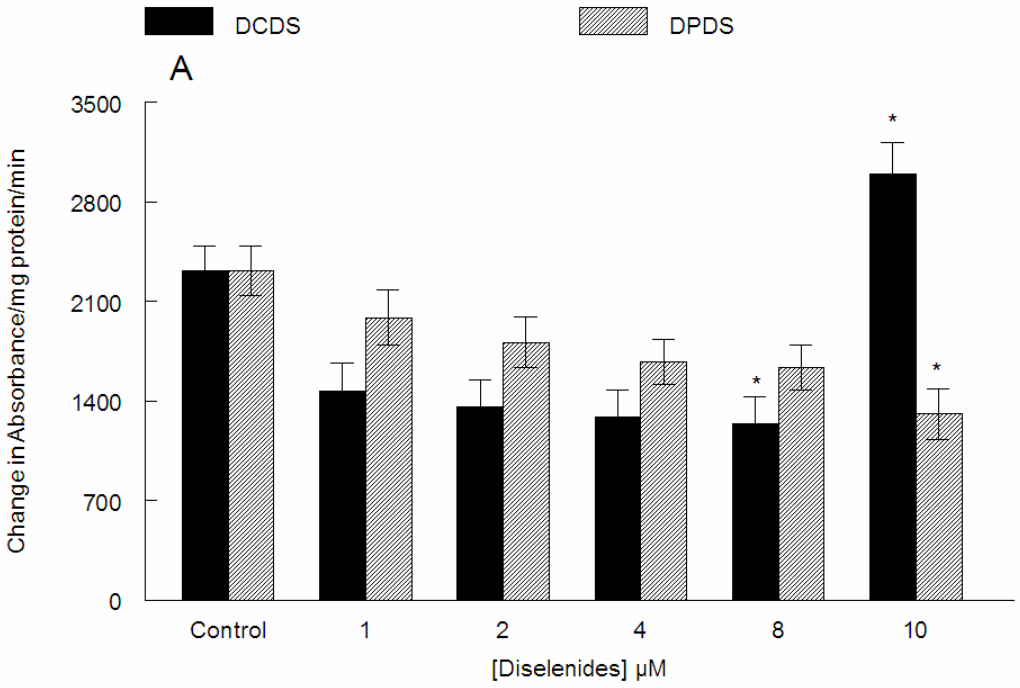


Figure 4: Effect of diselenides on renal LDH under different incubation conditions

Values are the means of three rats. *indicate significant difference in comparison with control. A) without lactate (P<0.005); B) without NAD (P<0.005); C) Without preincubation (P<0.005), and D) without all (P < 0.005). Values are expressed as change in absorbance/mg protein/minute.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3500
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700
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Change in Absorbance/mg protein/min
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*
Control
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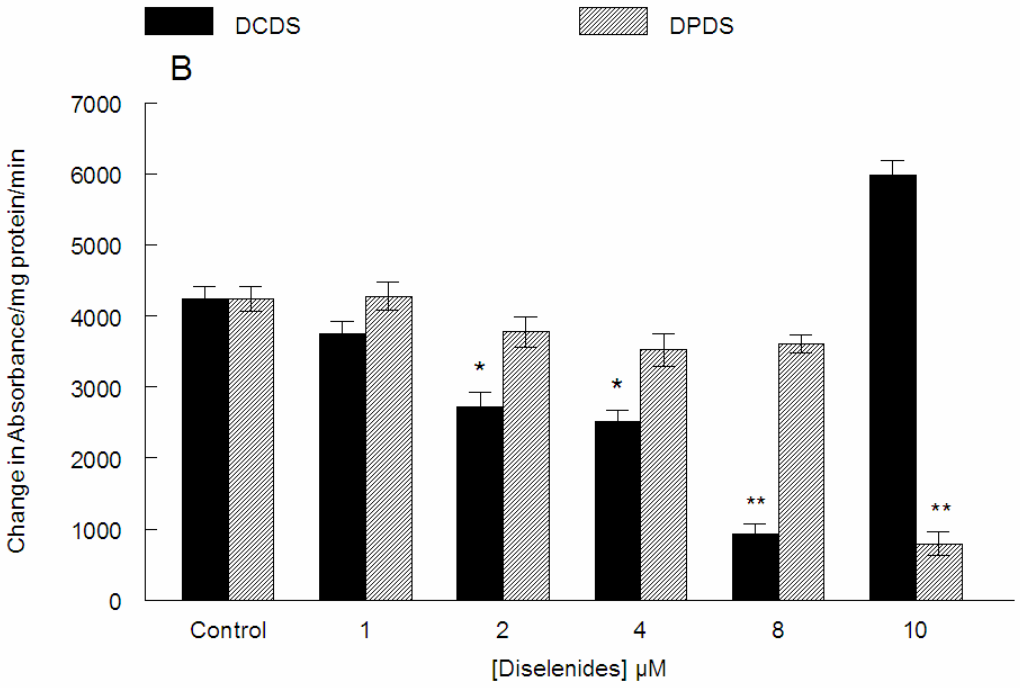
2
4
8
10
[Diselenides] µM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DPDS
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6000
5000
4000
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2000
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0
Change in Absorbance/mg protein/min
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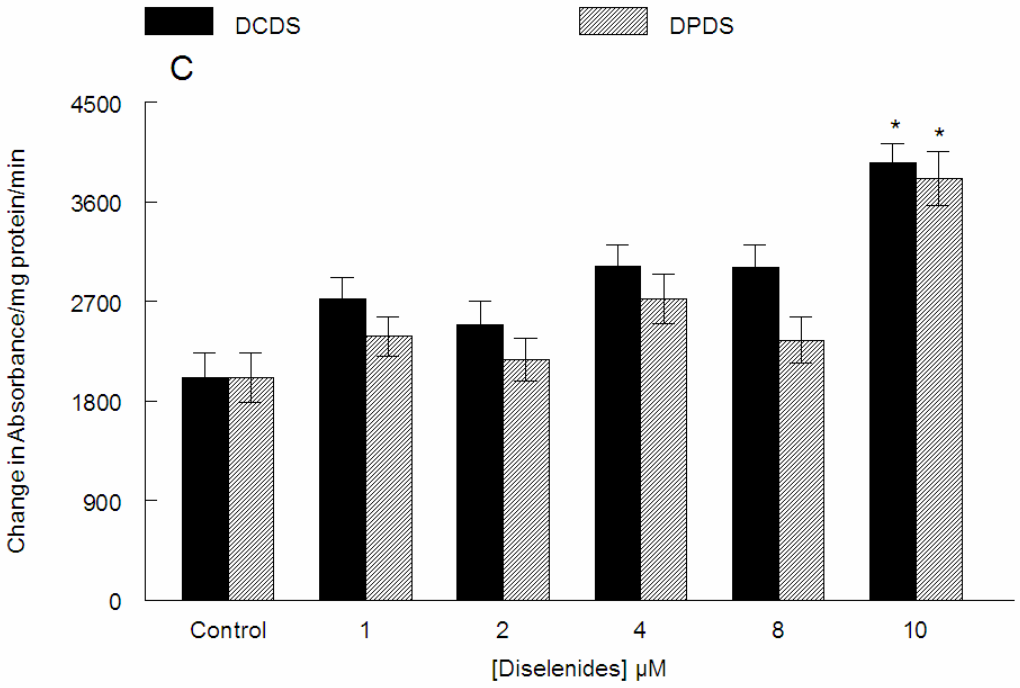
Control
1
2
4
8
10
[Diselenides] µM


DCDS
DPDS
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Change in Absorbance/mg protein/min
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Control
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[Diselenides] µM

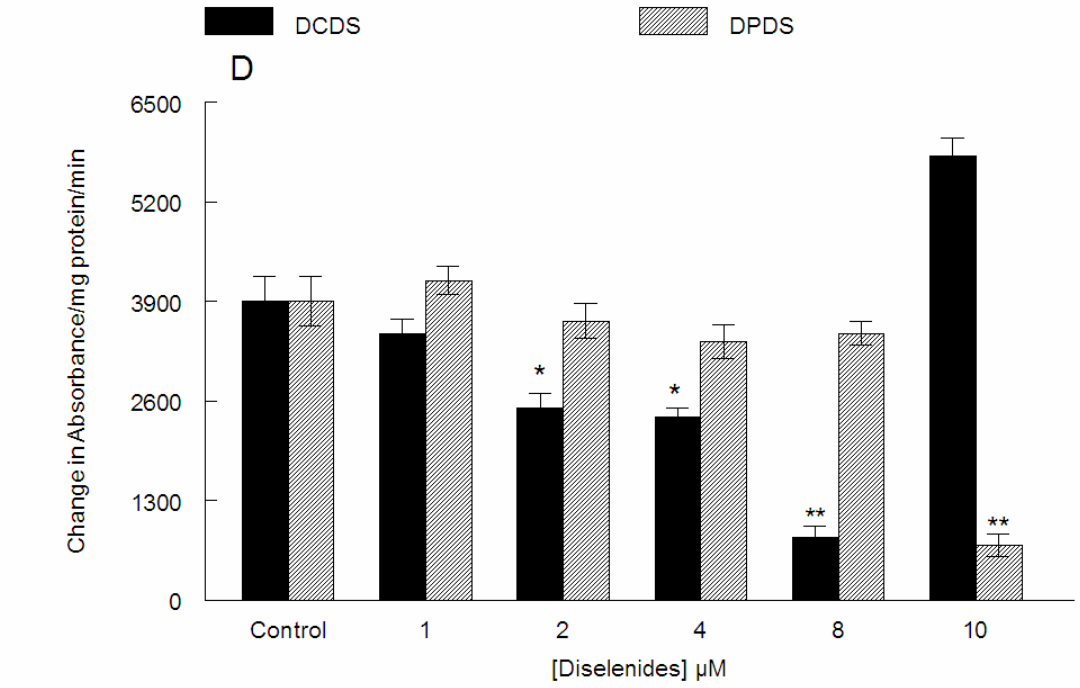
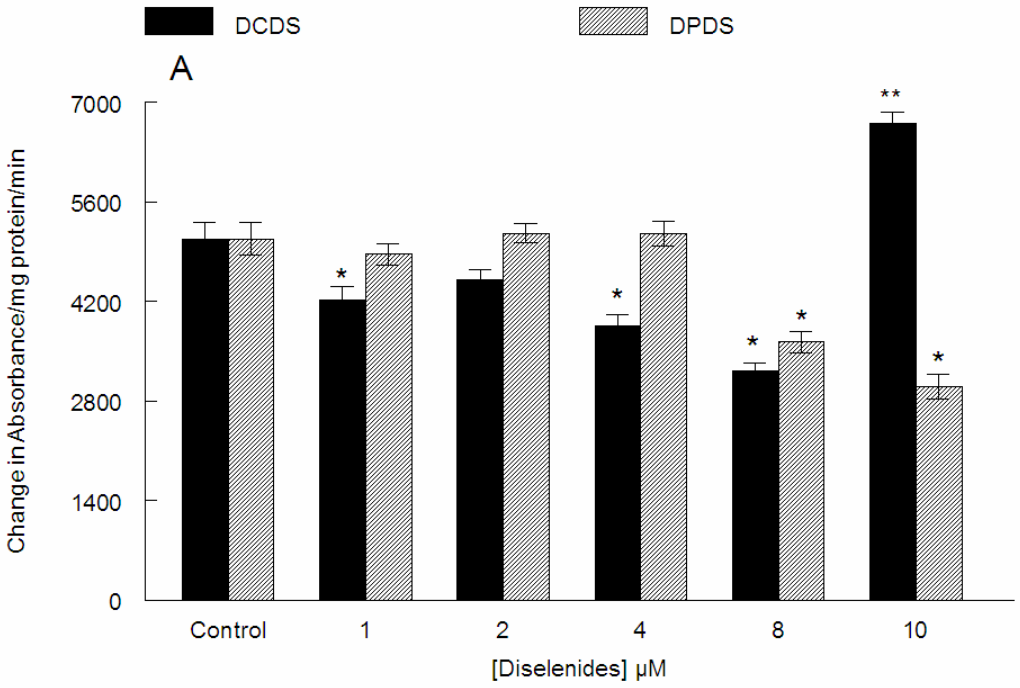


Figure 5: Effect of diselenides on hepatic LDH under different incubation conditions

Values are the means of three rats. *indicate significant difference in comparison with control. A) without lactate (P<0.0001); B) without NAD (P<0.0001); C) Without preincubation (P<0.0001), and D) without all (P < 0.0001). Values are expressed as change in absorbance/mg protein/minute.


DCDS
DPDS
A
7000
5600
4200
2800
1400
0
Change in Absorbance/mg protein/min
Control
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10
[Diselenides] µM
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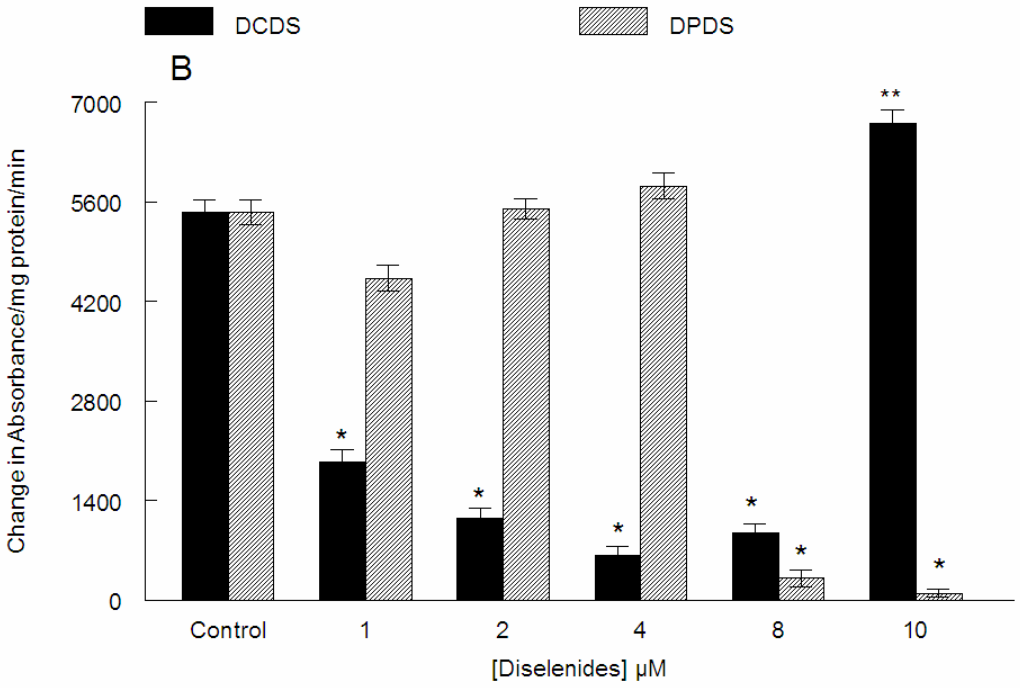
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Change in Absorbance/mg protein/min
Control
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[Diselenides] µM
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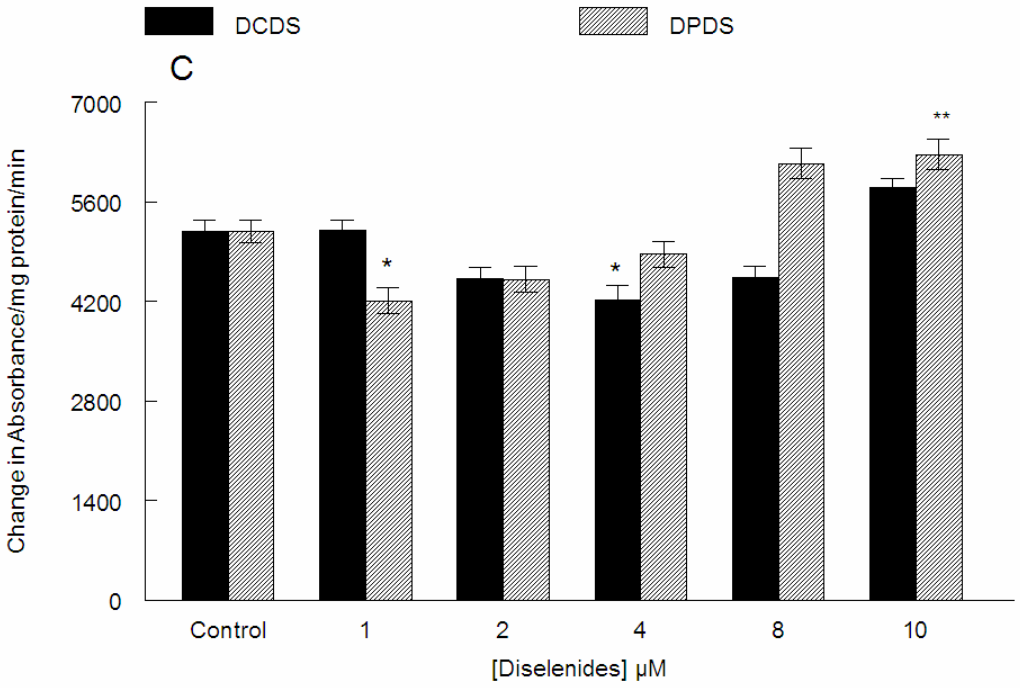
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Change in Absorbance/mg protein/min
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Control
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[Diselenides] µM

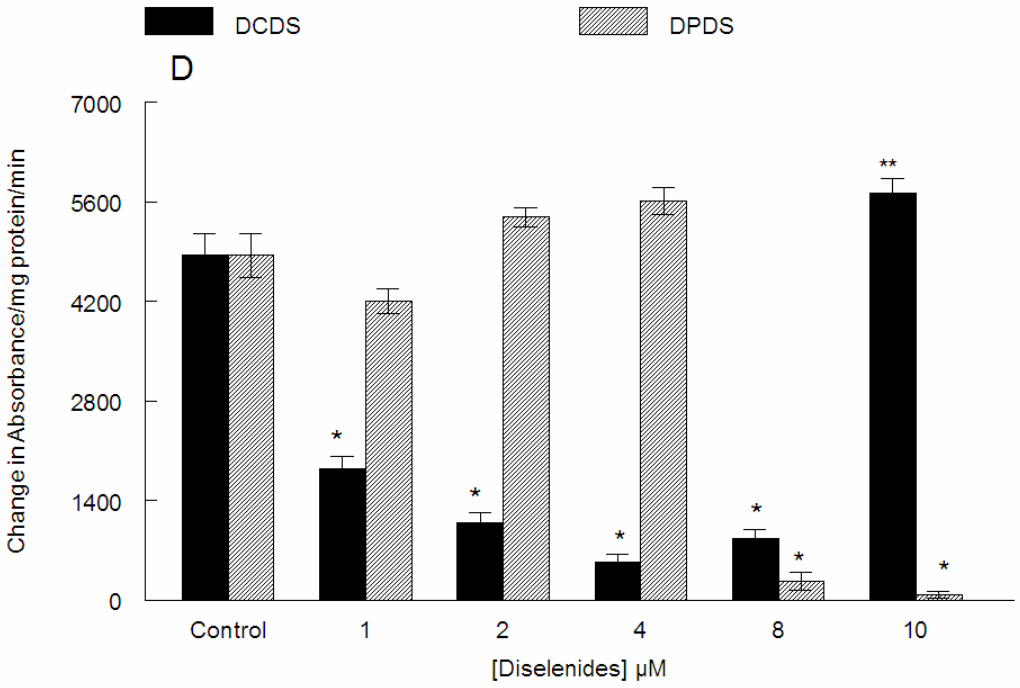
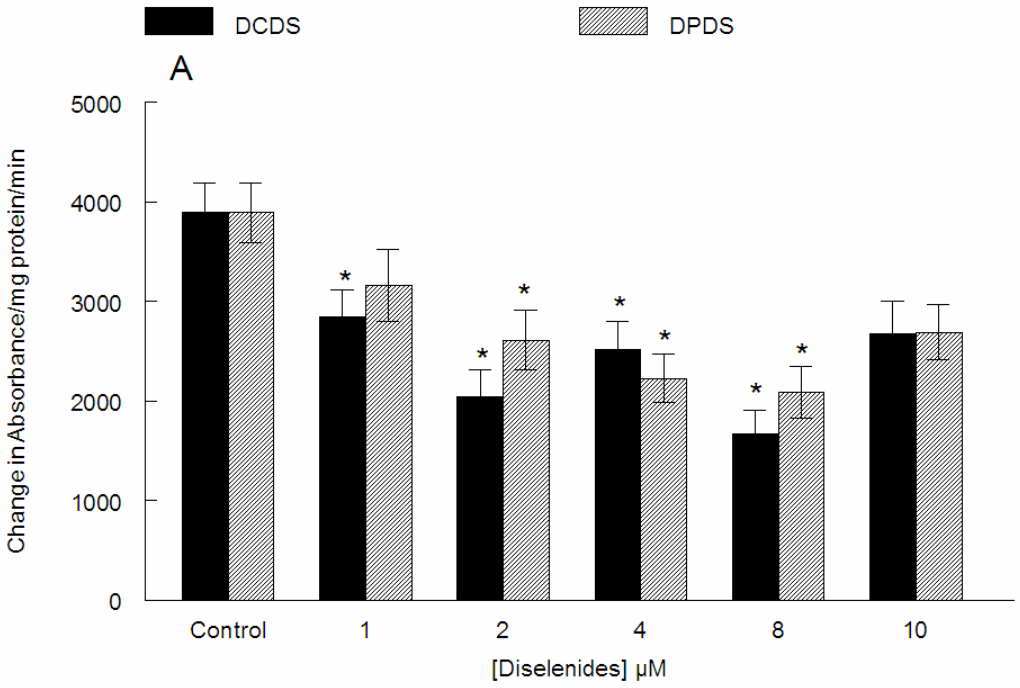
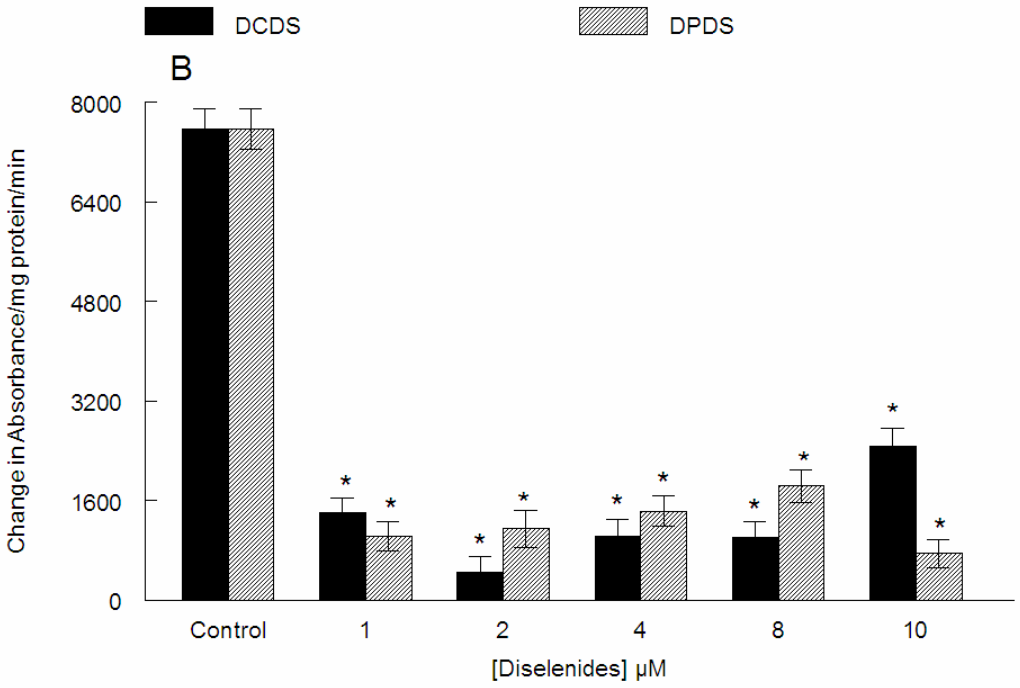
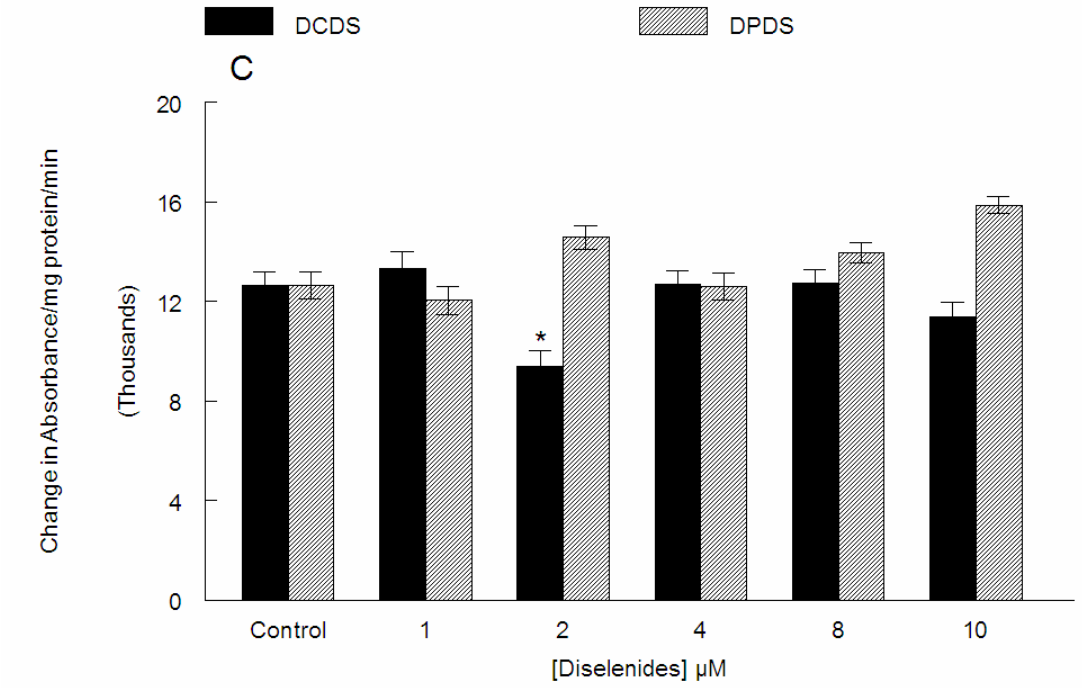


Figure 6: Effect of diselenides on cardiac LDH under different incubation conditions. Values are the means of three independent experiments. *indicate significant difference in comparison with control. A) without lactate (P<0.0001); B) without NAD (P<0.0001); D) Without preincubation (P<0.0001), and E) without all (P < 0.0001). Values are expressed as change in absorbance/mg protein/minute.


DCDS
DPDS
A
5000
4000
3000
2000
1000
0
Change in Absorbance/mg protein/min
Control
1
2
4
8
10
[Diselenides] µM


DCDS
DPDS
B
8000
6400
4800
3200
1600
0
Change in Absorbance/mg protein/min
Control
1
2
4
8
10
[Diselenides] µM


DCDS
DPDS
C
Change in Absorbance/mg protein/min
(Thousands)
20
16
12
8
4
0
*
Control
1
2
4
8
10
[Diselenides] µM

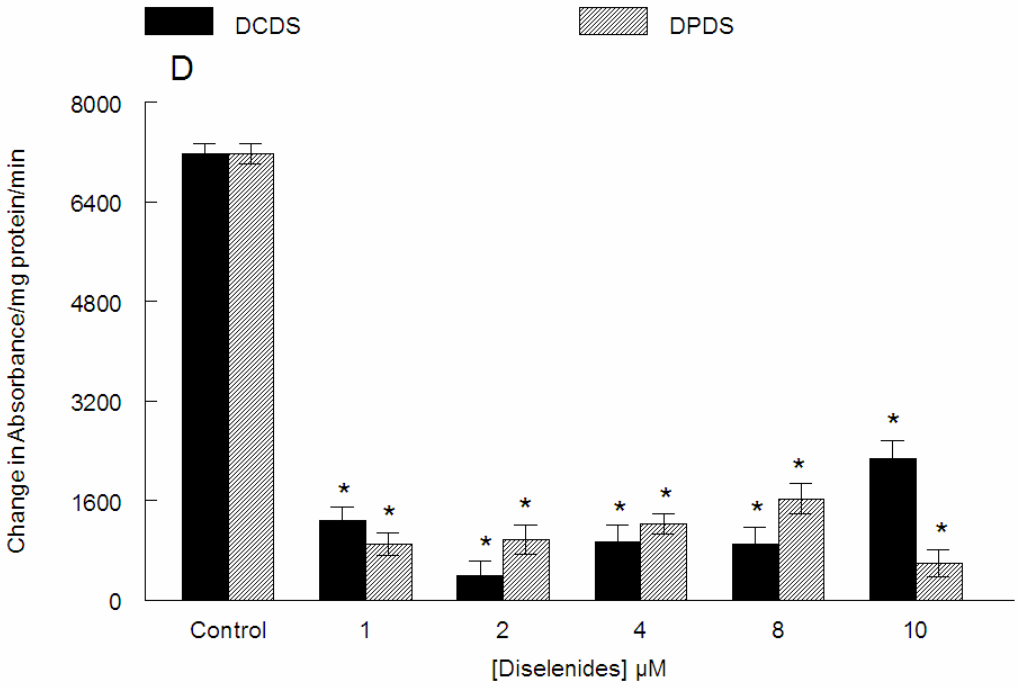


Figure 7: Effect of diselenides on Purified cardiac LDH under different incubation conditions. Values are the means of three independent experiments. *indicate significant difference in comparison with control. A) without lactate (P<0.0001); B) without NAD (P<0.0001); C) Without preincubation (P<0.0001), and D) without all (P < 0.0001). Values are expressed as change in absorbance/mg protein/minute.

3.2 *In Vivo* Studies

3.2.1 Third Manuscript

EFFECT OF ORAL ADMINISTRATION OF DIPHENYL DISELENIDE ON ANTIOXIDANT STATUS AND ACTIVITY OF DELTA AMINOLEVULINIC ACID DEHYDRATASE AND ISOFORMS OF LACTATE DEHYDROGENASE IN STREPTOZOTOCIN INDUCED DIABETIC RATS

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(Submitted to Cell Biology and Toxicology)

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ABSTRACT

Diabetes was induced by the administration of streptozotocin (STZ) (45 mg/kg, intravenous) in male albino rats and was treated with oral administration of diphenyl diselenide (DPDS) dissolved in soya bean oil. We observed that there was a significant reduction in blood glucose levels of STZ induced diabetic rats treated with DPDS in relation to untreated STZ diabetic group. This effect of DPDS was accompanied by a reduction in the levels of glycated proteins. In addition, DPDS was able to significantly restore the levels of Vitamin C and GSH levels (in liver and kidney tissues), which were significantly decreased in different tissues in STZ-treated rats. Similarly, treatment with DPDS was able to markedly abolish the increased levels of TBARS that were observed in STZ-induced diabetic group. Finally, the inhibition of catalase, delta aminolevulinic acid dehydratase (ð-ALAD) and isoforms of lactate dehydrogenase (LDH) accompanied with hyperglycemia were prevented by DPDS in all tissues examined. Hence in comparison with our earlier report, we conclude that irrespective of the route of administration and delivery vehicle, diphenyl diselenide can be considered as an anti-diabetogenic agent by exhibiting anti-hyperglycemic and antioxidant properties.

Keywords: Diphenyl diselenide, diabetes, streptozotocin, lactate dehydrogenase, delta aminolevulinic acid dehydratase.

INTRODUCTION

Diabetes mellitus, an endocrine disorder, has been described as the metabolic disorder with the highest rates of prevalence and mortality world-wide [Barcelo and Rajpathak, 2001]. Attention has been drawn to the theory that oxidative stress is an important pathogenic constituent in diabetic endothelial dysfunction and is directly related to the cause of complications in chronic diabetes [Baynes, 1991]. In fact, reportS have indicated that diabetics and experimental animal models of diabetes exhibit high oxidative stress due to persistent and chronic hyperglycemia, thereby depleting the activity of the antioxidative defense system and thus promote free radicals generation [Hammers *et al*., 1991], which in turn, elicit free radical induced tissue damage, ultimately resulting in complications that often characterize diabetes mellitus disorders [Oberley, 1988]. Potential sources of increased free radical production in diabetes include autoxidation of glucose, activation of leukocytes, and increased transition metal availability [Wolff *et al*., 1991]. In this context, antioxidant therapies have been shown to improve glycemia levels in diabetic individuals [Sharma *et al*., 2000]. Hence there is an increased interest in the assessment of antioxidant agents as a possible chemotherapeutic candidate in the management of diabetes mellitus.

Selenium is an essential element with physiological antioxidant properties, appearing as a selenocysteine, a structural component of several enzymes involved in peroxide decomposition, including glutathione peroxidase [Arteel and Sies, 2001, Zhao and Holmgren, 2002] and phospholipid hydroperoxide glutathione peroxidase [Ursini *et al*., 1982]. Organic forms of selenium have been suggested as possible antioxidant agents because they exhibit glutathione peroxidase-like activity [Arteel and Sies, 2001]. Interestingly, Selenium-containing compounds have been proved to have insulin-mimetic properties *in vitro* [Ezaki, 1990] and *in vivo* [McNeill *et al*, 1991] and exert antioxidant characteristics in diabetic animal models [Mukherjee, 1998]. In fact, diabetes mellitus has

been related to alteration in the homeostasis of selenium [Simonoff and Simonoff, 1991]. In addition, selenium containing organic compounds (generally referred to as organochalcogens) also retard the lipoperoxidation induced by a variety of oxidants [Rossato *et al*., 2002]. Of particular importance is that recent data from our laboratory have demonstrated that diphenyl diselenide (DPDS) and ebselen when administered *subcutaneously* and in Tween solution to streptozotocin (STZ) diabetic rats demonstrated anti-hyperglycemic and antioxidants effect [Barbosa *et al*., 2006].

However, earlier report by our group has shown that diphenyl diselenide exhibit differential toxicity in animal models [Nogueira *et al*., 2003]. In this regard, DPDS was more toxic in mice than rats when administered by the intraperitoneal route (LD50 210 and 1200 *í*mol/kg, respectively) and had no acute toxic effects when administrated by the subcutaneous route [Nogueira *et al*., 2004]. In addition, the vehicle used in the administration of organoselenides can equally affect their interactions with physiological and biochemical parameters in animal models [Britto *et al*., 2006]. Hence, *in vivo* pharmacology potency of organoselenium compounds largely depends on the concentration, the chemical form and also on the route of administration of such compound [Nogueira, *et al.,* 2004; Whanger, 2004].

In view of the foregoing, we decided to explore the possibility of an altered pharmaco-potency of DPDS when given orally and in soya bean oil instead of subcutaneous and in Tween solution to STZ treated rats as demonstrated in our earlier report [Barbosa *et al*., 2006]. Our intention was to determine the antioxidative and antihyperglycaemic effect of DPDS in STZ-induced diabetic rat models. In addition, since thiol containing enzymes are the prime targets of organoselenium compounds, the present study would also evaluate the effect of oral administration of DPDS on the activity of an important thiol containing protein, delta aminolevulinic acid dehydratase, which has been associated with

hyperglycaemia and organodiselenides toxicity *in vivo* [Folmer *et al*., 2002]. These indices would be evaluated in different tissues of the rats treated with either STZ or DPDS or both.

2. MATERIALS AND METHODS

2.1. Chemicals

ð-Aminolevulinic acid (ð-ALA), *p*-dimethylaminobenzaldehyde, streptozotocin (STZ), reduced glutathione (GSH), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA) were obtained from Sigma (St. Louis, MO, U.S.A.). Diphenyl diselenide was synthesized according to the literature methods [Paulmier, 1986]. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with their assigned structures. The chemical purity of diphenyl diselenide (99.9%) was determined by GC/HPLC. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Animals

Adult male Wistar rats weighing 180–200 g were used for the experiments. All rats received food (Guabi, Ribeirao Preto, SP, Brazil) and water *ad libitum* and were kept on a 12 h ligth/12 h dark cycle, in a room with the temperature regulated to 21–25°C and humidity at roughly 56%. The animals were used accordingly to guidelines of the Committee on Care and use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil.

2.3. Diabetes induction

Diabetes was induced by a single intravenous injection of streptozotocin (STZ) 45 mg/kg), diluted in 0.1M citrate-buffer (pH 4.5). Control rats received an equivalent amount of the

buffer. Diabetic state was checked 72 h after induction with STZ. Blood samples were taken from the tail vein of rats to determine glucose levels by an automatic autoanalyzer (GLUCOTREND®). Animals were considered diabetic when blood glucose levels exceeded above 250 mg/dl.

2.4. Treatment

The animals were randomly divided into the following groups: (group 1) control; (group 2) diphenyl diselenide; (group 3) streptozotocin (STZ); and (group 4) streptozotocin + diphenyl diselenide. Groups 2 and 4 were administered with diphenyl diselenide by gavage at the dose of 3 mg/kg (once a day) for 35 days after the administration of STZ. The organoselenium compounds were dissolved in 1ml of soya bean oil. Control rats were similarly orally administered with soya bean oil. At the end of the experimental period, diabetic rats and the corresponding control animals were anesthetized with ether and euthanized. Rats were fasted 12 h prior to the euthanasia.

2.5. Tissue preparation

Tissue samples were quickly removed, placed on ice and homogenized in cold 50mM Tris–HCl pH 7.4. The homogenate was centrifuged at 4000×*g* for 10 min to yield the low-speed supernatant (S1) fraction that was used for biochemical assays. For all analysis, protein content was determined by the method of Lowry *et al*., [1951], using bovine serum albumin as the standard.

2.6 Biochemical analyses

Plasma activities of AST (aspartate aminotransferase), ALT (alanine aminotransferase) and urea, creatinine, cholesterol and triglyceride levels were determined using commercial Kits (Labtest, Minas Gerais, Brazil).

2.7 Glycated proteins

The levels of glycated-hemoglobin were determined in plasma by using commercial Kits (Labtest, Minas Gerais, Brazil).

2.8 Antioxidant defense systems

2.8.1 Vitamin C content

Hepatic, spleenic, and renal vitamin C levels were determined colorimetrically as described by Jacques-Silva *et al*., [2001]. Briefly, Proteins were precipitated in 10 volumes of a cold 4% trichloroacetic acid solution. An aliquot of the sample at a final volume of 1 ml of the solution was incubated for 3 h at 38 °C then 1ml H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using a color reagent containing 4.5 mg/ml dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/ml) at 520 nm. The content of ascorbic acid is related to tissue amount (μmol ascorbic acid/g wet tissue).

2.8.2. Non-Protein SH level

Blood (erythrocytes), hepatic renal and spleenic reduced glutathione (GSH) content was estimated using Ellman's reagent after deproteinization with TCA (5% in 1 mmol/EDTA) by the method of Ellman, (1959).

2.8.3 TBARS Assay in Tissues

Rats were euthanized under mild ether anesthesia and the hepatic, spleenic and renal tissues were rapidly dissected, placed on ice and weighed. Tissues were immediately homogenized in cold 10mM Tris–HCl, pH 7.5 (1/10, w/v) with 10 up-and-down strokes at approximately 1200 rev/min in a Teflon-glass homogenizer. The homogenate was centrifuged for 10 min at 4000×*g* to yield a pellet that was discarded and a low-speed supernatant (S1). Productions of TBARS were determined as described by Ohkawa *et al*. (1979). Briefly to an aliquot of 100 µl of S1, 1.52% (final concentration) sodium dodecyl sulphate (SDS); 500 µl acetic acid/HCl (pH 3.4) and 0.25% (final concentration) thiobarbituric acid (TBA) were sequentially added. The mixture was incubated at 100^0C for 1hr and the TBARS produced were measured at 532 nm and the absorbance was compared to that of a standard curve obtained using malondialdehyde (MDA).

2.9. Enzymes activity

2.9.1. δ-ALA-D

Hepatic, renal and spleenic ð-ALA-D activity was assayed according to the method of Sassa, [1982] by measuring the rate of product porphobilinogen (PBG) formation except that 84mM potassium phosphate buffer, pH 6.4 and 2.4mM ALA were used. The reaction was started 10 min after the addition of enzyme by adding the substrate (ð-ALA). Incubations were carried out for 1 h (liver), 2 h (kidney) and 2 h (spleen) at 37^0C. The reaction product was determined using modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of 6.1×104M−1 for the Ehrlich-PBG salt.

2.9.2. LDH Activity

LDH activity was monitored spectrophotometrically by the rate of increase in absorbance at 340 nm at 37°C resulting from formation of NADH. The assay medium contained 21 mM of glycine-KOH buffer (pH 10), 50 mM of lactate (pH 6.8), 30µL of liver (150 to 200µg of protein) or spleen (150 to 200 µg of protein) or kidney (120 to 180 µg of protein), and the reaction was started by adding neutralized NAD^+ to provide a final concentration of 0.25 mM.

2.9.3. Catalase Activity

Catalase activity was assayed spectrophotometrically by the method of Aebi [1984], which involves monitoring the disappearance of H_2O_2 in the homogenate presence at 240 nm. Enzymatic reaction was initiated by adding an aliquot of the homogenized tissue and the substrate (H_2O_2) to a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.0. The enzymatic activity was expressed in units (1 U decomposes 1 µmol of H_2O_2 per minute at pH 7 at 25 °C).

3. Statistical analysis

All values obtained are expressed as mean±S.E.M The data were analyzed by two-way ANOVA analyses of variance followed by Duncan's multiple range tests where appropriate.

4.0 RESULTS

4.1 Body weight

Two-way ANOVA [(with or without STZ) X (with or without DPDS)] revealed that there was a marked reduction in body weight gain in all streptozotocin treated rats in comparison to the control group [($p < 0.05$ for weeks 1 and 3) and ($p < 0.001$ for weeks 2, 4 and 5)]

(Fig. 1). It is noteworthy that the reduction in body weight of diabetic rats was not significantly prevented by diphenyl diselenide, and also, the administration of diphenyl diselenide alone did not change the body weight gain of rats. Further, in comparison to group 3, the apparent increase in body weight gain in group 4 animals was not significant ($P > 0.1$). In fact, further statistical analysis shows that for all weeks, there was no significant interaction among the groups ($P > 0.05$).

4.2 Plasma glucose levels

Two-way ANOVA [(with or without STZ) X (with or without DPDS)] showed that for days 2, 15 and 35, the blood glucose in all STZ treated rats (groups 3 and 4) were significantly ($P < 0.001$) higher in comparison with control animals. As we can observe (Table 1), DPDS caused a significant decrease in the glucose levels of group 4. This anti-hyperglycemic effect of diphenyl diselenide was evident on the 35th day after STZ administration and the blood glucose levels of group 4 was more than 50% lower than that of group 3. In fact, a further statistical comparison showed that the difference in blood glucose between groups 3 and 4 is significant ($P < 0.001$) and two-way ANOVA yielded a significant interaction on days 15 and 35 ($p < 0.000001$).

4.3 Biochemical analysis

For each biochemical parameters investigated, a two-way ANOVA was carried out (Table 2). Plasma triglyceride was markedly increased in diabetic rats when compared to control group ($p < 0.0001$). Diphenyl diselenide treatments restored triglyceride levels in STZ treated rats. Although there was no significant difference between the values of triglycerides in group 1 and 4 ($p > 0.3$), however, there exist a significant difference when groups 3 and 4 were compared ($P < 0.001$). Further, a two-way ANOVA yielded a

significant interaction ($p < 0.00001$). In addition, rats treated with either STZ, DPDS or both exhibited a marked elevated level of cholesterol when compared to the control rats ($p < 0.01$). In fact, two-way ANOVA yielded a significant interaction ($p < 0.1$). However, as observed in table 2, AST, ALT, urea, and creatinine were not significantly modified by STZ and/or DPDS treatments. In fact, two-way ANOVA did not yield any significant interaction ($p > 0.5$) for any of the parameters (AST, ALT, urea and creatinine).

4.4 Antioxidant defenses

4.4.1. Vitamin C levels

Vitamin C levels were markedly decreased in both hepatic ($p < 0.001$) and renal ($p < 0.0001$) tissues of STZ treated rats when compared to control. On the other hand, STZ treatment did not cause a marked decrease in the vitamin C level in the spleen. However, treatment with diphenyl diselenide normalized Vitamin C levels in all tissues of STZ treated rats (Table 3). It would be observed that DPDS caused a marked increase in vitamin C level in spleen of group 2 animals ($p < 0.1$). Two-way ANOVA yielded a significant interaction for hepatic ($p < 0.0001$) and renal ($p< 0.00001$) and a non significant interaction for spleenic vitamin C.

4.4.2 GSH levels

Hepatic GSH levels were significantly modified by STZ treatment ($p < 0.001$) when compared to group 1 animals. However, STZ treatment did not significantly decrease both renal and spleenic GSH levels ($p > 0.05$). Treatment with DPDS promoted *per se* a significant increase on hepatic, renal and spleenic GSH levels compared to control group (Table 3). However, two-way ANOVA yielded a significant interaction for only liver ($p < 0.00001$) and not for kidney ($p > 0.5$) and spleen ($p > 0.2$).

4.4.3 TBARS

TBARS level in both liver and kidney were markedly elevated ($p < 0.0001$) in STZ treated animals in comparison with control rats (Table 3). However, there was no marked difference in the TBARS level of spleenic tissues of either STZ or DPDS treated or both. It would also be observed that, in relation to group 1 animals, DPDS caused a decrease in the level of TBARS in both liver and kidney in group 4 animals, nevertheless, the levels of hepatic and renal TBARS level in group 4 is still significant ($p < 0.01$). In fact, two-way analysis yield a significant interaction for both liver ($p < 0.01$) and kidney ($p<0.00001$).

4.5 Enzymes activity

4.5.1. δ-ALA-D

Generally, the activity of ð-ALA-D was significantly reduced in the liver ($p < 0.0001$), kidney ($p < 0.0001$) and spleen ($p < 0.05$) of STZ treated rats when compared to the control group (Table 4). It is also noteworthy that diphenyl diselenide caused a marked reduction in the activity of ð-ALA-D in both liver and kidney of group 2 rats ($p < 0.0001$). Nonetheless, DPDS significantly reverted the inhibition of ð-ALA-D imposed by STZ treatment in group 4 rats and this reversion is significant for both hepatic and renal ð-ALA-D ($p < 0.0001$) when compared with group 3 animals. In fact, two-way ANOVA yielded a significant interaction for both the liver and kidney enzyme activity ($p < 0.00001$)

4.5.2 Lactate dehydrogenase

STZ treatment caused a significant [($p < 0.001$ for liver; $p < 0.0001$ for both kidney and spleen)] decreased in all isoforms of lactate dehydrogenase (LDH) evaluated when compared to control group (Table 4). Treatment of diabetic rats with diphenyl diselenide significantly improved LDH activity to control levels. Two-way ANOVA [(with or without

STZ) X (with or without DPDS)] analysis of the activity of all isoforms of LDH yielded a significant interaction ($p < 0.00001$). The inhibition is significant because as we can observe, STZ caused a marked decrease of the enzyme activity and DPDS treatment was able to markedly relieve the inhibition. In fact, activity of the hepatic LDH in group 4 rats was significantly increased in relation to group 1 ($p < 0.0001$). In addition, for all isoforms, the increase in LDH activity in group 4 rats was significant ($p < 0.0001$) when compared to groups 3 rats.

4.5.3 Catalase

Hepatic catalase activity was significantly ($p < 0.05$) elevated in diabetic rats when compared to the control group (Table 4). However, in both the kidney and spleen, there were no differences in catalase activity of group 3 animals when compared to group 1 animals. In fact, two-way ANOVA [(with or without STZ) X (with or without DPDS)] yielded a significant ($p < 0.05$) interaction for only the hepatic enzyme.

5.0 Glycated-hemoglobin levels

Total glycated-hemoglobin concentrations in STZ treated rats (group 3) were significantly ($p < 0.001$) higher than group 1 rats (Figure 2). In addition, diphenyl diselenide treatment caused a significant ($p < 0.0001$) reduction in glycated proteins levels in diabetic rats. However, DPDS was able to restore the elevated level of glycated proteins to a non-significant level in group 4 animals when compared to control group. Further statistical analysis yielded a significant ($p < 0.00001$) interaction.

DISCUSSION

Several reports have shown that DPDS have potent antioxidant activity both *in vitro* and *in vivo* [Nogueira *et al*., 2004, Mugesh *et al*., 2001], and recently we demonstrated that subcutaneous administration of DPDS in Tween was able to temporarily reduce hyperglycemia with a modification in the antioxidant status of STZ induced diabetic animal [Barbosa *et al*., 2006]. In fact, in relation to our earlier report, the present study shows that irrespective of the route of administration and vehicle solution DPDS exerts antihyperglycaemic activity. Additionally, we observed in Table 1 that DPDS significantly ($p<0.001$) reduced blood glucose level in STZ treated rats on days 15 and 35 when compared to untreated STZ diabetic rats. Therefore we can conclude that in relation to our earlier report [Barbosa *et al*., 2006], both oral and subcutaneous administration of DPDS shows similar antihyperglycaemic potency in relation to diminution of blood glucose.

Earlier studies have demonstrated that diabetes in humans is characterized by progressive β-cell failure [Cnop *et al*., 2005]. In fact, type 1 diabetes is caused by an autoimmune assault against the pancreatic β-cells, with invasion of the islets by mononuclear cells in an inflammatory reaction termed "insulitis," leading to progressive loss of most β-cells [Kloppel *et al*., 1985]. β-cell death in the course of insulitis has been inked to cascades of events such as direct contact with activated macrophages and T-cells, and/or exposure to soluble mediators secreted by these cells, including cytokines, nitric oxide (NO), and oxygen free radicals [Eizirik and Mandrup-Poulsen, 2001]. In addition, apoptosis can be activated and/or modified by extracellular signals, intracellular ATP levels, phosphorylation cascades, and expression of pro and anti-apoptotic genes [Eizirik and Mandrup-Poulsen, 2001]. On the other hand, the pathogenesis of type 2 diabetes is more variable, comprising different degrees of β-cell failure relative to varying degrees of insulin resistance, often associated with obesity and insulin secretion defects [Kahn, 2003].

Consequently, a progressive decrease of β-cell function leads to glucose intolerance, characterizing type 2 diabetes that aggravates with time [Cnop *et al*., 2005; Kahn, 2003] as it does in rodent models of the disease [Rhodes 2005]. Interestingly, streptozotocin (an analogue of N-acetyl-beta-D-gucosamine, (GlcNAc)), the diabetogenic agent employed in this study, have been reported to produce beta-cell necrosis in the rat possibly by inhibiting N-acetyl-beta-D-glucosaminidase (O-GlcNAcase), the enzyme that removes O-GlcNAc from protein [Ganda *et al*., 1976; Konrad *et al*., 2001]. Therefore, STZ increases pancreatic islet O-linked protein glycosylation and causes beta-cell death by inhibiting O-GlcNAcase, thereby suggesting that the mechanism of STZ diabetogenic toxicity may be related to beta cell death [Ganda *et al*., 1976; Konrad *et al*., 2001]. Hence, STZ may be an excellent diabetogenic agent that can simulate the diabetic conditions found in human subjects.

Reports have shown that hyperglycemia causes an excessive non-enzymatic glycation of protein structures, with marked inactivation of enzymes; increased lipid peroxidation and changes in the antioxidant defense systems [Caballero *et al*., 2000; Morgan *et al*., 2002]. However DPDS significantly reduced the levels of glycated proteins and this may be related to its antidiabetic properties (fig 2).

Additionally, selenium compounds have been reported to possess insulin-like properties [Ezaki, 1990; Stapleton, 2000]; and they reduce blood glucose by stimulating glucose uptake in insulin-responsive tissues consequent from phosphorylation reactions of the β-subunit of the insulin receptor and other downstream components of the insulin signaling pathways like IRS 1, IRS 2, S6 kinase and MAPK [Stapleton *et al*., 1997; Ezaki, 1990], and also positively affects the expression and activity of enzymes associated with both carbohydrate and fatty acid metabolism *in vitro* [Berg *et al*., 1995; Stapleton *et al*., 1997]. In this regard, we observed that in diabetic rats, there was an elevated level of triglyceride which was significantly modified by DPDS (Table 2). Additionally, this may equally help

to explain in parts the attendant marked increase in cholesterol level in groups 3 animals. However, the significant increase in cholesterol level in group 2 animals may be difficult to explain within the scope of the current study. In any case, an evaluation of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, and creatinine shows that there were no marked differences (in relation to group 1 rats) in the levels of these markers in rats treated with STZ, DPDS or both.

Tissue damage associated with hyperglycaemia has been related to oxidative stress [Ratner, 2001]. Hence we evaluated enzymic and non-enzymic antioxidant status of liver, kidney and spleen, since these are targeted organs in diabetic complications [Ratner, 2001]. With respect to non-enzymic antioxidants such as GSH and Vitamin C, the levels of these antioxidants were observed to be significantly decreased in the liver and kidney but not the spleen and were restored by diphenyl diselenide treatment, indicating that diphenyl diselenide besides acting as an antioxidant may promote an increase in antioxidant systems. In addition, diphenyl diselenide, orally administered to diabetic rats shows a potential in modifying the level of hepatic and renal thiobarbituric acid reactive species (TBARS). This is in agreement with our earlier observation that DPDS therapy reduces the development of pathological conditions associated with oxidative stress by exerting peroxynitrite scavenger properties in other experimental models of diabetes [Barbosa *et al.*, 2006].

Catalase is an antioxidant enzyme that reduces hydrogen peroxides formed in cellular metabolism. In the present study, the level of catalase was significantly elevated only in the hepatic tissues of STZ treated rats, and this was modified by DPDS. It is noteworthy that there were discrepancies in the levels of liver antioxidant enzymes reported in diabetic rats [Wohaieb and Godin, 1987; Ozkaya *et al*., 2002].

Organoselenium compounds can interact directly with low molecular thiols, oxidizing them to disulfides. Reduced cysteinyl residues from proteins can also react with these

compounds, which may cause, in the case of enzymes, the loss of their catalytic activity. For instance, ð-aminolevulinate dehydratase or porphibilinogen synthase (ð-ALA-D), one of the enzyme evaluated in this study, is a sulfhydryl- containing enzyme that is extremely sensitive to oxidizing agents [Farina *et al*., 2003]. This enzyme catalyzes the asymmetrical condensation of two molecules of 5-aminolevulinic acid, to form porphibilinogen, an intermediate in tetrapyrol biosynthesis. Hence, this enzyme plays a fundamental role in most living aerobic organisms by participating in heme biosynthesis. Furhermore we earlier observed that the activity of delta ALA-D have been inhibited in cases of diabetes [Folmer *et al*., 2002]. In the present study, we observed that STZ significantly caused an inhibition in the activity of ð-ALA-D in both liver and kidney and DPDS was able to significantly relieve this inhibition caused by STZ.

Another thiol ontaining enzyme evaluated in this study is lactate dehydrogenase (LDH), which reversibly converts pyruvate and NADH, H+ into lactate and NAD^+. The two isoforms are labeled H (heart) and M (muscle), (formerly referred to as B and A respectively) and their ratio varies between cell types. The LDH isoform ratio has been proposed to indicate the metabolic state of cells: it is believed that the M isoform favors lactate production while the H isoform favors pyruvate production [Stambaugh and Post, 1966]. Diabetes is generally characterized by a loss of insulin activity, culminating in profound changes in glucose metabolism (in glycolysis) and gene expression [Yechoor, *et al*., 2002], and these changes were associated with altered levels of NAD^+ and NAD^+/NADH, H^+ ratios in the metabolism [Wahlberg, *et al*., 2000]. In fact, since there is only a limited amount of NAD^+ in the cell, NADH, H^+ requires reoxidation back into NAD^+ in order that glycolysis may continue. The rate of conversion of the NADH, H^+/NAD^+ ratio is regulated by the activities of LDH The data presented in Figure 4 shows that STZ caused a markedly decrease in the activity of LDH in all the tissues (liver, kidney

and spleen) studied. However, administration of DPDS significantly reversed this inhibition in the different isoforms of LDH studied. We may speculate that the decreased level of LDH in STZ treated rats may result from the need to regulate the extent of cellular acidosis which are markedly increased in diabetes [Yechoor, *et al*., 2002; Wahlberg, *et al*., 2000].

It has been reported that reduced lipid peroxidation and improved antioxidant status may be one mechanism by which dietary treatment contributes to the prevention of diabetic complications [Armstrong *et al*., 1996]. Improvement of antioxidant status in diabetes is likely to have a number of benefits. Evidence is accumulating to support the idea that there is a close relationship between the processes of oxidation and glycation, and that antioxidants (particularly vitamin C and vitamin E) may specifically inhibit glycation of proteins [Davie *et al*., 1992; Ceriello *et al*., 1991]. Clinical trials of antioxidant therapy to prevent ischemic heart disease in nondiabetic subjects are currently beginning. It has been speculated that diabetic patients may represent a group who would achieve particular benefit in terms of reduced atherosclerosis from antioxidant supplementation. Indeed, Morel and Chisolm, [1989] have shown that antioxidant treatment can inhibit both lipoprotein oxidation and cytotoxicity in diabetes.

Notwithstanding, however, the present study demonstrated that the treatment of diabetic rats with oral administration of DPDS in soya bean oil at 3mg/kg exerted a considerable hypoglycemic effect. Also, DPDS could improve on the antioxidant status as well as relieve the inhibition of thiol containing enzymes such as delta aminolevulinic acid dehydratase and lactate dehydrogenase in the liver, kidney and spleen of streptozotocin induced diabetes.

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TABLES

Table 1

Glucose levels (mg/dl) in STZ and DPDS treated rats.

Days	Control	DPDS	STZ	STZ+DPDS
3	88±9	80±15	547±30[b]	555±43[c]
15	89±8	79±13	487±39[c]	297±35[b,e]
35	87±8	81±17	461±43[c]	192±34[b,e]

Data are expressed as means ± S.E.M. of seven animals.

[b,c]Significantly different from the control group ([b]) $p < 0.001$ and ([c]) $p < 0.0001$.

[e]Significantly different from the diabetic group (ANOVA/Duncan, $p < 0.001$).

Table 2

Biochemical parameters of STZ and DPDS treated rats measured on the 35th day.

Parameters	Control	DPDS	STZ	STZ+DPDS
ALT*	111.33±14.01	135±34.83	169.3±83.3	165.3±72.8
AST*	114.3±9.02	119.3±8.5	159.3±72.7	165±65
UREA#	50±2.65	46.7±6.5	57±9.8	57.5±9.3
TRIGL#	115.3±23.4	100.3±37.87	374.5±41.8[c]	133.8±45.3[e]
CHOL#	71±6.2	108.3±25.5[a]	100.5±16.5[a]	101±20.02[a]
CREAT#	0.153±0.02	0.163±0.11	0.168±0.07	0.163±0.09

Data are expressed as means ± S.E.M. seven animals.

Data of renal lipid markers are presented as mg/dl.

* Data of enzyme activities are presented as U/l.

[a,c]Significantly different from the control group ([a]) $p < 0.01$ and ([c]) $p < 0.0001$.

[e]Significantly different from the diabetic group (ANOVA/Duncan, $p < 0.001$).

Table 3

TBARS, Vitamin C and non protein thiol levels in STZ and DPDS treated rats measured on the 35th day

Parameters	Control	DPDS	STZ	STZ+DPDS
TBARS[A]				
Liver	457.6±25.5	580±34.02	825±221[c]	651.5±25.5[a,d]
Kidney	297.7±43.02	348.7±34.02	505.2±34[c]	358.9±30.6[a,e]
Spleen	631.1±73.1	660±163.3	666.8±165	637.9±56.1
Vitamin C[B]				
Liver	409.93±14.73	446.33±34.67	208±19.93[b]	433.33±95.3[f]
Kidney	303.3±32.07	277.3±19.07	201.9±18.2[c]	288.6±15.6[f]
Spleen	457.6±34.67	513.93±31.2	465.4±51.1	496.6±19.9
GSH[D]				
Liver	19.58±1.01	22.61±1.38[b]	10.32±0.69[b]	22.72±1.60[b,f]
Kidney	8.78±0.69	11.07±0.27[b]	8.67±0.80	10.69±1.28[b,e]
Spleen	7.45±0.74	7.08±0.69	6.6±0.69	6.92±0.96

Data are expressed as means±S.E.M. of seven animals.

[A]Unit of TBARS is µM MDA/hr/g tissue.

[B]Unit of Vit C is µg AA/g tissue

[D]GSH levels are presented as µmol/g of tissues.

[a,b,c] Different from the control group, ([a]) $p < 0.01$; ([b]) $p < 0.001$ and ([c]) $p < 0.0001$.

[d,e,f] Different from the diabetic group ([d]) $p < 0.01$; ([e]) $p < 0.001$ and ([f]) $p < 0.0001$.

Table 4: Activity of catalase, LDH and ð-ALA-D in STZ and DPDS treated rats measured on the 35th day

Parameters	Control	DPDS	STZ	STZ+DPDS
Catalase[A]				
Liver	64484±6648	63985±11634	84760±4986[a]	55343±16620[e]
Kidney	45081±13841	40533±14829	56549±19772	48047±15818
Spleen	53640±11283	42542±12948	61778±8693	50126±12948
Lactate Dehydrogenase[B]				
Liver	2954±63.8	3047.5±23.5[b]	1439±54.7[b]	3111±16.7[c,f]
Kidney	906.5±17.5	881.3±21.23	476.7±24.04[c]	897.4±29.14[f]
Spleen	701.3±10.3	694±24.3	303.4±41.8[c]	696±19.77[f]
ð-Aminolevulinic Acid Dehydratase[D]				
Liver	0.695±0.06	0.579±0.025[b]	0.379±0.02[c]	0.618±0.07[a,f]
Kidney	0.475±0.042	0.396±0.024[b]	0.346±0.03[c]	0.483±0.018[f]
Spleen	0.159±0.02	0.148±0.008	0.140±0.01	
0.146±0.0104				

Data are expressed as means ± S.E.M. of seven animals.

[A]Unit of Catalase is µmol H_2O_2 /mg protein/min

[B]Values are expressed as change in absorbance/mg protein/minute.

[D]Unit of ALAD is nmol of PBG/µg protein/hr

[a,b,c] Different from the control group, ([a]) $p < 0.01$; ([b]) $p < 0.001$ and ([c]) $p < 0.0001$.

[e,f] Different from the diabetic group ([e]) $p < 0.001$ and ([f]) $p < 0.0001$..

FIGURES

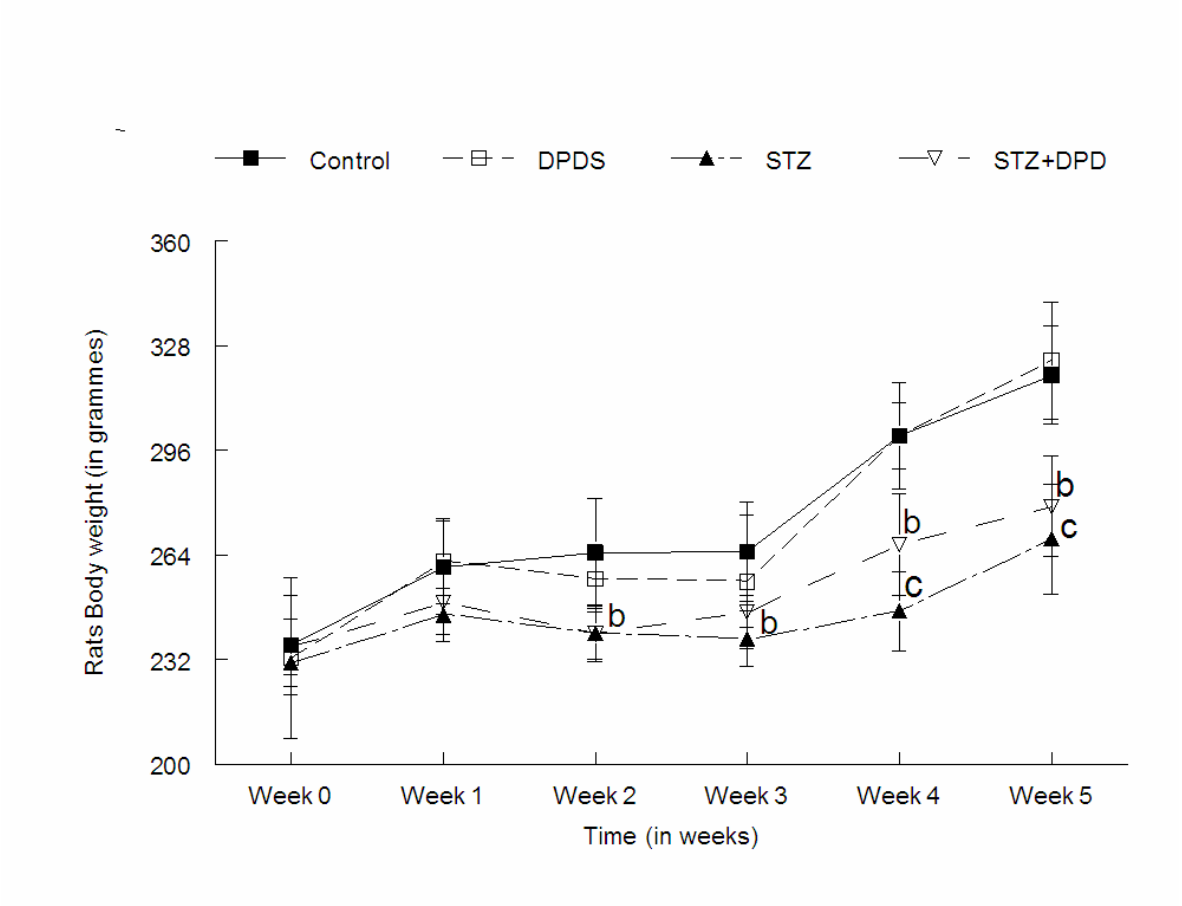

Fig.1. Body weight (means ± S.E.M., n = 7) of STZ-induced diabetic rats treated with diphenyl diselenide. [b,c] indicate significant different from the control group ([b]) $p < 0.001$ and ([c]) $p < 0.0001$.

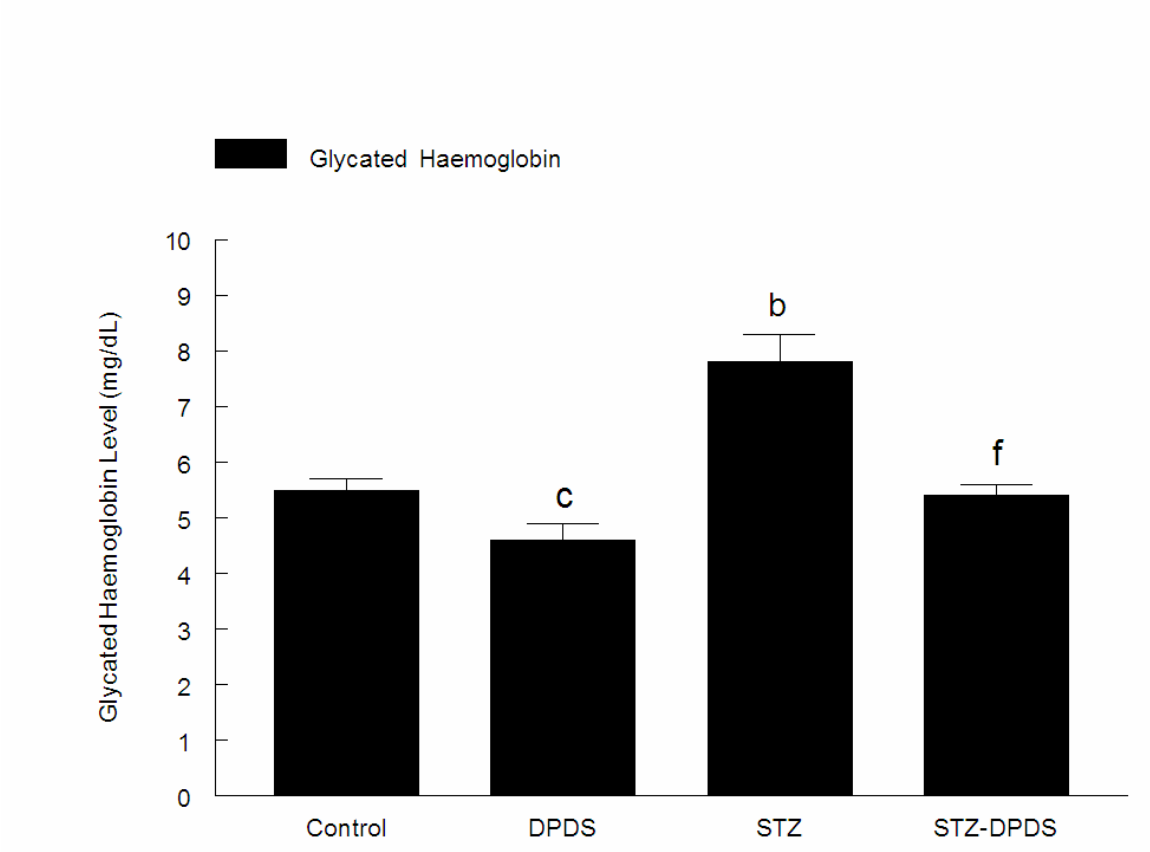

Fig. 2. Plasma concentration of glycated-hemoglobin in STZ-induced diabetic rats treated with diphenyl diselenide. Values are means ± S.E.M. of six animals. [b,c] indicate significant different from the control group ([b]) $p < 0.001$ and ([c]) $p < 0.0001$; whereas ([f]) denotes significant different from the diabetic group at $p < 0.0001$.

3.2.2 Fourth Manuscript

DIPHENYL DISELENIDE AND STREPTOZOTOCIN DID NOT ALTER CEREBRAL GLUTAMATERGIC AND CHOLINERGIC SYSTEMS BUT MODULATE ANTIOXIDANT STATUS AND Na^+/K^+-ATPase ACTIVITY IN DIABETIC RATS

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(Manuscript under preparation)

DIPHENYL DISELENIDE AND STREPTOZOTOCIN DID NOT ALTER CEREBRAL GLUTAMATERGIC AND CHOLINERGIC SYSTEMS BUT MODULATE ANTIOXIDANT STATUS AND Na^+/K^+-ATPase ACTIVITY IN DIABETIC RATS

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ABSTRACT

Neuronal malfunction is a characteristics feature of diabetic mellitus. The present study therefore sought to evaluate the effect of diphenyl diselenide (DPDS) on the antioxidant status, sodium pump, cholinergic and gutamatergic system in the rat brain of streptozotocin (STZ) induced diabetes. The results show that although STZ evoke a significant diminution on the antioxidant status and activity of Na^+/K^+-ATPase, the activity of acetylcholineesterase and glutamate uptake and release was not altered. However, DPDS was able to markedly restore the observed imbalance in cerebral antioxidant status and also relieve the inhibition of Na+/K+-ATPase caused by streptozotocin. Hence, we conclude that DPDS is a potential candidate in the management of neuronal dysfunction that often accompanied complications associated with diabetic hyperglycaemia.

Keywords: Brain, glutamate uptake, glutamate release, acetylcholinesterase, Na+/K+-ATPase, delta aminolevulinic acid deydratase, lactate dehydrogenase

INTRODUCTION

Diabetes mellitus is a serious metabolic disorder characterized by hyperglycemia resulting from impairment in insulin production and is associated with long-term complications affecting the eyes, kidneys, heart and nerves (Gispen and Biessels, 2000; McCall, 1992), causing a variety of functional and structural disorders in the central and peripheral nervous systems (Biessels *et al*., 1994). In addition, electrophysiological and structural abnormalities of the brain in diabetic patients suggest that cognitive functions may be impaired in diabetes mellitus (Gispen and Biessels, 2000). Moderate impairment of learning and memory has been observed in adults with diabetes mellitus and this have been related to oxidative lipid and protein damage in the brain, constituting long term complications, morphological abnormalities and memory impairments (Fukui *et al*., 2001). The increased oxidative stress in diabetes produces oxidative damage in rat brain. Enhanced formation of oxygen free radicals occurs in tissues during hyperglycemia (Baydas et al., 2002). These oxidant radicals contribute to increased neuronal death through protein oxidation, DNA damage, and peroxidation of membrane lipids (Hawkins and Davies, 2001).

Glutamate, the main excitatory neurotransmitter in the mammalian brain, has important roles in several physiological and pathological events (Ozawa *et al*., 1998). Glutamatergic neurotransmission is achieved through ionotropic (ligand-gated ion channels) and metabotropic (G proteins-coupled) receptors. Specifically, the *N*-methyl-daspartate (NMDA) ionotropic glutamate receptor subtype seems to be crucial in plasticity processes associated with normal brain function (Izquierdo and Medina, 1997; Ozawa et al., 1998). However, overstimulation of the glutamatergic system, as observed when glutamate concentration in the synaptic cleft increases, may be neurotoxic. Glutamate neurotoxicity is

implicated in acute neurological disorders as well as in neurodegenerative diseases (Lipton and Rosenberg, 1994; Ozawa et al., 1998; Price, 1999).

Na +/K +-ATPase is a transmembrane enzyme primarily responsible for the active transport of sodium and potassium in mammalian cells [Glynn, 1985]. This protein is greatly expressed by neurons, consuming 30-60% of brain ATP store and it maintains the electrical potential necessary for excitability of this tissue. Since many metabolic pathways serve as targets for modification in diabetes mellitus, (Greene et al., 1987; Winegrad, 1987) with some of them resulting either in a lower concentration of the active assembled enzyme in the plasma membrane or in the development of altered physico-chemical parameters, this enzyme can also be influenced indirectly. These structural or dynamic changes indirectly influence the structure, dynamics, and interactions of the enzyme, resulting in enzyme activity changes. A definitive answer to the crucial question about the molecular mechanism of involvement of Na+/K+-ATPase in diabetes mellitus would represent a key step in the development of the proper treatment. (Zolese et al., 1997)

Acetylcholinesterase (Ache) is a marker from the cholinergic system. Ache is a serine hydrolase that belongs to the esterase family within higher eukaryotes (Massoulie et al., 1993). This enzyme is more abundant in the central nervous system and it is an important regulatory enzyme that controls the transmission of nerve impulses across cholinergic synapses by hydrolyzing the excitatory transmitter acetylcholine (Massoulie et al., 1993; Taylor and Radic, 1994; Muller et al., 2002). Ache is important for toxicological and pharmacological interests because it is a target for various cholinergic toxins, such as therapeutically active compounds to combat neurodegenrative diseases (Karczmar, 1998).

Selenium is a trace element that exerts certain insulin-like actions on the glucose homeostasis of diabetic rats (Becker *et al*., 1996; Kimura, 1996). Selenium has a large number of biological functions in the human organism. The most important and known

action is its antioxidant effect because it forms selenocysteine, part of the active center of the glutathione peroxidase enzyme GSH-Px (Chappuis and Poupon, 1991; Levander and Burk, 1994). Recently, our laboratory (Nogueira et al., 2001, 2002) has demonstrated that diphenyl diselenide modifies the functionality of the glutamatergic system *in vitro* and *in vivo*. In addition, DPDS has also been shown to inhibit Na+, K+ ATPase (Kade *et al*., 2008). However, data on the effect of artificially induced hyperglycaemic conditions on these neurological markers are still scanty. Moreso, the mechanisms leading to complex morphological, metabolic, and functional changes in the central and peripheral nervous system in diabetes mellitus are multiple and not completely understood. While the pathogenesis of peripheral neuropathy has been the subject of extensive research (Vinik, et al., 2000; Zochodne, 1999), disorders in brain functioning have not been fully explored yet. Hence the present study was designed to investigate alterations in antioxidant status, as well as cholinergic, glutamatergic as well as the sodium pump in STZ diabetic models.

MATERIALS AND METHODS

2.0. Chemicals

ð-aminolevulinic acid (ð-ALA), *p*-dimethylaminobenzaldehyde, streptozotocin (STZ), reduced glutathione (GSH), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA) were obtained from Sigma (St. Louis, MO, U.S.A.). Diphenyl diselenide was synthesized according to the literature methods [Paulmier, 1986]. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with their assigned structures. The chemical purity of diphenyl diselenide (99.9%) was determined by GC/HPLC. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.1. Animals

Adult male Wistar rats weighing 180–200 g were used for the experiments. All rats received food (Guabi, Ribeirao Preto, SP, Brazil) and water *ad libitum* and were kept on a 12 h ligth/12 h dark cycle, in a room with the temperature regulated to 21–25°C and humidity at roughly 56%. The animals were used accordingly to guidelines of the Committee on Care and use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil.

2.2. Diabetes induction

Diabetes was induced by a single intravenous injection of streptozotocin (STZ) 45 mg/kg), diluted in 0.1M citrate-buffer (pH 4.5). Control rats received an equivalent amount of the buffer. Diabetic state was checked 72 h after induction with STZ. Blood samples were taken from the tail vein of rats to determine glucose levels by an automatic autoanalyzer (GLUCOTREND®). Animals were considered diabetic when blood glucose levels exceeded above 250 mg/dl.

2.3. Treatment

The animals were randomly divided into the following groups: (1) control; (2) diphenyl diselenide; (3) streptozotocin (STZ); and (4) streptozotocin + diphenyl diselenide. Groups 2 and 4 were administered with diphenyl diselenide by gavage at the dose of 3 mg/kg (once a day) for 35 days after the administration of STZ. The organoselenium compounds were dissolved in 1ml of soya bean oil. Control rats were similarly orally administered with soya bean oil. At the end of the experimental period, diabetic rats and the corresponding control animals were anesthetized with ether and euthanized. Rats were fasted 12 h prior to the euthanasia.

2.4. Tissue preparation

Brain tissues were quickly removed, placed on ice and homogenized in cold 50mM Tris–HCl pH 7.4. The homogenate was centrifuged at 4000×*g* for 10 min to yield the low-speed supernatant fraction that was used for the determination of thiobarbituric acid reactive species (TBARS), catalase (CAT), delta aminolevulinic acid dehydratase (ð-ALA-D), Vitamin C (Vit C), glutathione (GSH) and lactate dehydrogenase (LDH). For all analysis in this study, protein content was determined by the method of Lowry *et al*., (1951), using bovine serum albumin (BSA) as the standard.

2.5. Antioxidant defense systems

2.5.1. Vitamin C content

Cerebral vitamin C levels were determined colorimetrically as described by Jacques-Silva *et al*., [2001]. Briefly, Proteins were precipitated in 10 volumes of a cold 4% trichloroacetic acid solution. An aliquot of the sample at a final volume of 1 ml of the solution was incubated for 3 h at 38 °C then 1ml H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using a color reagent containing 4.5 mg/ml dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/ml) at 520 nm. The content of ascorbic acid is related to tissue amount (μmol ascorbic acid/g wet tissue).

2.5.2. Non-protein thiol levels

Cerebral non-protein thiol content was estimated using Ellman's reagent after deproteinization with TCA (5% in 1 mmol/EDTA) as described by Ellman, (1959).

2.5.3. TBARS Assay in Tissues

Rats were euthanized under mild ether anesthesia and the hepatic, spleenic and renal tissues were rapidly dissected, placed on ice and weighed. Tissues were immediately homogenized in cold 10mM Tris–HCl, pH 7.5 (1/10, w/v) with 10 up-and-down strokes at approximately 1200 rev/min in a Teflon-glass homogenizer. The homogenate was centrifuged for 10 min at 4000×*g* to yield a pellet that was discarded and a low-speed supernatant (S1). Productions of TBARS were determined as described by Ohkawa *et al*. (1979). Briefly to an aliquot of 100 µl of S1, 1.52% (final concentration) sodium dodecyl sulphate (SDS); 500 µl acetic acid/HCl (pH 3.4) and 0.25% (final concentration) thiobarbituric acid (TBA) were sequentially added. The mixture was incubated at 100^0C for 1hr and the TBARS produced were measured at 532 nm and the absorbance was compared to that of a standard curve obtained using malondialdehyde (MDA).

2.6. Enzymes activity

2.6.1. δ-ALA-D

Cerebral ð-ALA-D activity was assayed according to the method of Sassa, [1982] by measuring the rate of product porphobilinogen (PBG) formation except that 84mM potassium phosphate buffer, pH 6.4 and 2.4mM ALA were used. The reaction was started 10 min after the addition of enzyme by adding the substrate (ð-ALA). Incubations were carried out for 3 h at 37^0C. The reaction product was determined using modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of 6.1×104M−1 for the Ehrlich-PBG salt.

2.6.2. Lactate dehydrogenase (LDH) Activity

Cerebral LDH activity was monitored spectrophotometrically by measuring the rate of increase in absorbance at 340 nm at 37°C resulting from formation of NADH. The assay medium contained 21 mM of glycine-KOH buffer (pH 10), 50 mM of lactate (pH 6.8), 30µL of brain (150 to 200µg of protein) and the reaction was started by adding neutralized NAD^+ to provide a final concentration of 0.25 mM.

2.6.3. Catalase Activity

Catalase activity in the brain was assayed spectrophotometrically by the method of Aebi [1984], which involves monitoring the disappearance of H_2O_2 in the homogenate's presence at 240 nm. Enzymatic reaction was initiated by adding an aliquot of the homogenized tissue and the substrate (H_2O_2) to a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.0. The enzymatic activity was expressed in units (1 U decomposes 1 µmol of H_2O_2 per minute at pH 7 at 25 °C).

2.7. Determination of acetylcholinesterase activity

Brain acetylcholinesterase activity was estimated by the method of Ellman *et al.* (1961), using acetylthiocholine iodide as a substrate. Whole rats brain were homogenized (1/5 w/v) in HEPES 25 mM buffer (pH 7.4) and the tissue homogenates were rapidly centrifuged at 20000×*g*, at 4°C for 30 min. The supernatants obtained were used for the determinations of enzymatic activity. The rate of hydrolysis of acetylthiocholine iodide is measured at 412 nm through the release of the thiol compound which, when reacted with 5'5'-dithio-bis(2-nitrobenzoic) acid (DTNB), produces the colour-forming compound thionitrobenzene (TNB).

2.8. Preparation of synaptosomes:

Rats were decapitated under mild ether and whole brain was removed and used to prepare synaptosomes on a discontinuous Percoll gradient according to Dunkley *et al*. (1988).

2.8.1. [^{3}H]Glutamate release by synaptosomes:

Determination of [^{3}H]glutamate released was accomplished according to the method described by Migues et al. (1999). The synaptosomal preparation was loaded with 0.25 µCi [^{3}H]glutamate (Amersham, specific activity 53 mCi/mmol, final concentration 5 µM) by pre incubation in Tris/HCl buffered salt solution (composition in mM: Tris/HCl 27, NaCl 133, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, Glucose 12, $CaCl_2$ 1.0) pH 7.4 (adjusted with HCl), for 15 min at 37°C. Aliquots of labeled synaptosomes (1.4 mg protein) were centrifuged at 16,000 g for 1 min. Supernatants were discarded, and the pellets were washed four times in Tris/HCl buffer by centrifugation at 16,000 g for 1 min (at 4°C). To assess the basal release of [^{3}H]glutamate, the final pellet was resuspended in Tris/HCl buffer and incubated for 60 s, at 37°C. Incubation was terminated by immediate centrifugation (16,000 g, 1 min, 4°C). Radioactivity present in supernatants and pellet was separately determined in a scintillation counter. The released [^{3}H]glutamate was calculated as a percentage of the total amount of radioactivity present in the synaptosomes at the start of the incubation period. K^+-stimulated [^{3}H]glutamate release was assessed as described for basal release, except for the fact that the incubation medium contained 40 mM KCl to induce synaptosomal depolarization.

2.8.2. [^{3}H]Glutamate uptake by synaptosomes:

Synaptosomal preparation was washed twice by suspending in 3 volumes of 0.3 M sucrose, in 15 mM Tris/acetate buffer (pH 7.4) and centrifuging at 35,000 g for 15 min. The final

pellet was suspended in 0.3 M sucrose, 15 mM Tris/acetate buffer (pH 7.4), and incubated in Tris/HCl buffer (composition in mM: Tris/HCl 27, NaCl 133, KCl 2.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, Glucose 12, $CaCl_2$ 1.0) pH 7.4 (adjusted with HCl), in the presence of [^{3}H]glutamate (final concentration 100 mM) for 1 min at 37°C. The reaction was stopped by centrifugation (16,000 g, 1 min, 4°C), and the pellets were washed three times in Tris/HCl buffer by centrifugation at 16,000 g for 1 min (at 4°C). Radioactivity present in pellet was measured in a scintillation counter. Specific [^{3}H]glutamate uptake was calculated as the difference between the uptake obtained in the incubation medium described above, and the uptake obtained with a similar incubation medium in which NaCl was replaced by choline chloride.

2.9. Na+-K+-ATPase

Cerebral Na+-K+-ATPase activity was measured as described by our group (Kade *et. al.*, 2008) except that there was no 10 minutes pre-incubation. Phosphate was measured according to Fiske and Subbarow (1925).

2.10. Behavioral test

2.10.1 Tail-immersion test

Thirty four days after STZ injections, rats were taken to a room adjacent to the test room for habituation and tested for somatic pain-threshold using the tail immersion test. Briefly, rats were gently picked up from their home cages, taken to the test room and the withdrawal latency of the tail to a temperature stimulus was measured by immersing the tip of the tail into a 55±0.5 ◦C water bath.

2.10.2 Open-field activity

For the open field test, rats were individually placed in a wooden cage square measuring 45 cm X 45 cm divided into nine squares using a tape. The number of crossing made across the internal squares as well as rearing (upright posture) were recorded over a period of 4 mins.

3. Statistical analysis

All values obtained are expressed as mean±SD. The data were analyzed by two-way ANOVA and MANOVA analyses of variance followed by Duncan's multiple range tests when appropriate. Differences between groups were considered to be significant when $p < 0.05$.

4.0 RESULTS

As part of the present study, we had recently reported that DPDS markedly diminished the elevated glucose level in STZ induced diabetes with concomitant significant reduction in the levels of glycated hemoglobin with attendant restoration of altered antioxidant status in diabetic rats (Kade *et. al.,* 2007). The results of various parameters evaluated are described below

4.1 Antioxidant status: TBARS, Vit C, and GSH

As presented in table 1, the level of thiobarbituric acid reactive species (TBARS) was markedly ($p < 0.0001$) elevated in STZ treated rats (group 3). However, treatment of diabetic rats with DPDS reduced the levels of TBARS. In fact, in comparison with group 3 animals, the level of reduction in group 4 animals was significant ($p < 0.001$). Also, it would be observed that STZ caused a noticeable diminution in the level of cerebral

glutathione (GSH) ($p < 0.005$); but treatment with DPDS evoked a significant sparing effect on the levels of GSH in the diabetic rat brain. Vitamin C level was markedly decreased ($p < 0.0001$) in STZ treated rats, and DPDS was able to restore the diminished level of vitamin C. In fact, in comparison with diabetic rats, the level of vitamin C in group 4 rats was significant higher than that of group 3 ($p < 0.005$). Two-way ANOVA yielded a significant interaction for cerebral TBARS ($p < 0.01$) and GSH ($p< 0.000001$) and vitamin C ($p < 0.000001$).

4.2 Enzymes activity

4.2.1 Catalase

Cerebral catalase activity was significantly ($p < 0.0005$) elevated in diabetic rats when compared to the control group (Figure 1). In fact, two-way ANOVA [(with or without STZ) X (with or without DPDS)] yielded a significant ($p < 0.05$) interaction. It is noteworthy that although DPDS did not restore the activity of catalase to control level, however, the reduction in catalase activity is significant when compared to the diabetic group ($p < 0.005$)

4.2.2 Lactate dehydrogenase

STZ treatment caused a significant ($p < 0.005$) decrease in the cerebral isoform of lactate dehydrogenase (LDH) when compared to control group (Figure 2). Treatment of diabetic rats with diphenyl diselenide significantly improved LDH activity to control levels. In fact, comparison of the level of LDH in both group 3 and 4 animals yielded a non significant difference ($p > 0.05$). Two-way ANOVA [(with or without STZ) X (with or without DPDS)] analysis of the activity of cerebral LDH yielded a significant interaction ($p < 0.05$).

4.2.3. δ-ALA-D

Generally, the activity of ð-ALA-D was significantly reduced in the brain of diabetic rats ($p < 0.0001$) when compared to the control group (Figure 3). Treatment of diabetic rats with DPDS completely restored the level of the enzyme activity.

4.2.4. Na^+/K^+-ATPase

As shown in figure 4, we observed that STZ caused a significant ($p < 0.0001$) diminution in the activity of cerebral Na^+/K^+-ATPase and that DPDS was able to relieve the inhibition of the enzyme activities resulting from STZ treatment. In fact, in comparison with diabetic rats (group 3), the activity of Na^+/K^+-ATPase was markedly ($p < 0.005$) elevated in group 4.

4.2.5. Acetylcholinesterase

Figure 5 shows the activity of actylcholinesterase in rats treated with STZ, DPDS or both. We can observe that there was no statistical difference in the activities of this cerebral enzyme subjected to the various treatments. In fact, two-way ANOVA [(with or without STZ) X (with or without DPDS)] analysis yielded a non significant interaction ($p > 0.2$).

4.3. Studies on glutamate uptake and release

Neither STZ treatment nor DPDS altered the glutamatergic system in the present studies. As we can see in figures 6-8; the there were no observed difference in either glutamate uptake, or glutamate released (both basal and stimulated) in all the animals in the groups (2, 3 and 4) when compared to control rats (group 1). Two-way ANOVA [(with or without STZ) X (with or without DPDS)] analysis yielded a non significant interaction [$p > 0.57$ for

glutamate uptake; $p > 0.72$ for glutamate released (basal); and $p > 0.49$ for glutamate released (stimulated)].

4.4. Behavioural studies

Behavioural studies on the rats in all the groups show that STZ caused an altered behaviour in diabetic rats. In figures 9 and 10, the number of crossings and rearing made by the diabetic rats (group 3) was significantly ($p < 0.0001$) lower in comparison with the control rats. However, DPDS treatment improved the comportment of the animals. When compared to diabetic rats (group 3), the number of crossings and rearing made by rats in group 4 is significantly higher ($p < 0.005$). Similarly, the time expended before diabetic rats (group 3) flicked their tail away from hot water (55^0C) was significantly higher when compared to control rats (group 1). DPDS treatment reduced the time expended in group 4 rats. In fact, the time difference between group 3 and group 4 rats is significant ($p < 0.005$)

DISCUSSION

Diphenyl diselenide is a promising candidate in the treatment of diabetic complications (Barbosa, *et al*., 2006). We have recently attributed the antihyperglycaemic properties of diphenyl diselenide to its antioxidant properties (Kade, *et al*., 2007). However, DPDS and other organochalcogenides are neurotoxic suggesting that the brain may be a potential target of organoselenium compounds (Nogueira *et al*. 2004, Britto *et al*. 2006, Prigol *et al*., 2007, Kade *et al*., 2008). In addition, diabetic complications have been associated with neurological disorders (Biessels et al., 1994). Therefore, it then becomes highly imperative that an evaluation of diabetic conditions vis-à-vis treatment with organodiselenides would involve evaluating neuronal processes.

In the present data, DPDS markedly restored the observed diminution in the level of vitamin C and GSH in STZ rats. In fact, in rats treated with only DPDS, the level of these antioxidants were observed to be markedly higher than in control. In addition, the level of TBARS was significantly higher in diabetic rats and DPDS was able to modify the elevated level of lipid peroxidation in diabetic rats. These results support our earlier observations that the major pharmacological potency of DPDS may be strongly related to its antioxidative properties (Nogueira *et al*., 2004). In fact, the activity of catalase, an antioxidant enzyme that reduces hydrogen peroxides formed in cellular metabolism, was significantly elevated the brain tissues of STZ treated rats, and this was modified by DPDS. It is noteworthy that there were discrepancies in the levels of antioxidant enzymes reported in diabetic rats [Wohaieb and Godin, 1987; Ozkaya *et al*., 2002].

The toxicity of organodiselenide has been has been related to it ability to interact with thiol groups on proteins. Hence, thiol containing proteins are more vulnerable to attack by organodiselenides. In addition, diabetic complications have been associated with impaired function of enzymes these thiol containing enzymes. Hence the activities of some of these critical cerebral sulphydryl enzymes were evaluated in this study and these include Na^+/K^+-ATPase, delta aminolevulinic acid dehydratase (ALAD), lactate dehydrogenase and acetylcholinesterase.

Diabetes induced by STZ provoke a significant reduction in membrane bound Na^+/K^+-ATPase in the brain, and that DPDS abolished such effects. Our results are in agreement with evidence presented by other investigators, showing that Na^+/K^+-ATPase activity is decreased in an experimental model of diabetes in rats [Ver et al., 1995; Oner, et al., 1997), a fact that may be an important factor in the pathogenesis of metabolic complications of the central nervous system in the diabetic state. However, the physiological significance of these alterations of brain Na^+/K^+-ATPase in diabetes remains to be clarified. A possible

mechanism to explain diabetes-induced inhibition of Na^+/K^+-ATPase is the well-documented increase in free radicals in diabetes [Noorouz-Zadeh et al., 1997; Haffner et al., 1995]. In this context, it has been demonstrated that elevated blood glucose concentration can induce generation of oxygen species [Obeley, 1988]. On the other hand, the structural properties [Chong et al., 1985] and lipid composition [Sandermann, 1978] of synaptosomal membrane are essential for the enzyme activity and lipid peroxidation and/or free radicals inhibit Na^+/K^+-ATPase activity in rat brain [Viani et al., 1991, Lees, 1993).

Delta-aminolevulinate dehydratase or porphibilinogen synthase (ð-ALA-D) is another cerebral sulfhydryl- containing enzyme that is extremely sensitive to oxidizing agents [Farina *et al*., 2003] and which has been inhibited in cases of diabetes [Folmer *et al*., 2002]. In the present study, we observed that STZ significantly caused an inhibition in the activity of cerebral ð-ALA-D and DPDS was able to significantly relieve this inhibition caused by STZ.

Likewise, cerebral isoform of lactate dehydrogenase (LDH), which reversibly converts pyruvate and NADH, H+ into lactate and NAD^+ was also investigated in the present study. Diabetes is generally characterized by a loss of insulin activity, culminating in profound changes in glucose metabolism (in glycolysis) and gene expression [Yechoor, *et al*., 2002], and these changes were associated with altered levels of NAD^+ and NAD^+/NADH, H^+ ratios in the metabolism [Wahlberg, *et al*., 2000]. In fact, since there is only a limited amount of NAD^+ in the cell, NADH, H^+ requires reoxidation back into NAD^+ in order that glycolysis may continue. The rate of conversion of the NADH, H^+/NAD^+ ratio is regulated by the activities of LDH. We observed that STZ caused a marked decrease in the activity of cerebral LDH. However, administration of DPDS significantly reversed this inhibition this isoforms of LDH. We may speculate that the decreased level of LDH in STZ treated rats

may result from the need to regulate the extent of cellular acidosis, which are markedly increased in diabetes [Yechoor, *et al*., 2002; Wahlberg, *et al*., 2000].

With regards to the activity of cerebral actylcholineesterase (AchE), the level was neither modified by STZ nor DPDS nor both (Figure 5). This result is consistent with reports by other authors. For example, Millind *et al*. (1995) and Striker *et al*. (1993) have reported that the kinetic properties of brain AchE, were not significantly altered by artificially induced diabetes.

Studies have shown that glutamate transporter located at either plasma or vesicular membrane contains reactive thiol groups in their structure and oxidation of these critical cysteinyl residues results in reduced uptake of glutamate (Trotti, *et al.,* 1999). Consequently, oxidant agents can produce neurotoxicity by increasing extracellular glutamate. In contrast, alterations of glutamate release by modulation of redox state are more complex (Zoccarato *et al*., 1995, 1999). In fact, thiol-oxidizing agents reduce K1-evoked glutamate release (Gilman *et al*., 1992; Zoccarato *et al*., 1995, 1999) while increase the basal release of glutamate (Gilman *et al*., 1992). Although *in vitro* as well as *in vivo* data (Nogueira et al., 2004) suggests that DPDS can modify glutamatergic system, this modification was not evident in the present study and may be related to the concentration of DPDS used. Also, STZ did not evoke any significant alteration in the glutamatergic system.

The open field test shows that the activity of the animals in STZ treated groups was significantly less compared to the control group. In addition, the tail flicking shows that the sensitivity of the STZ group was less compared to the control. We speculate that the observed differential behavioural may be related to a malfunction in neuronal system. However, while this may be related in part to the activity of Na^+/K^+-ATPase, the present study suggest that the behavourial alteration may not be related to the cholinergic and glutamatergic system.

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Table 1: TBARS level and non-enzymatic antioxidant defenses of STZ-induced diabetic rats treated with diphenyl diselenide

Parameters	Control	DPDS	STZ	STZ+DPDS
TBARS	387.8±8.0	389.8±6.5	891.6±8.5[a]	573.8±12.1[a,b]
GSH	21.5±1.24	24.6±1.3[a]	11.8±0.9[a]	23.7±1.1[a,b]
VIT C	331.4±10.8	329±7.4	232.6±5.7[a]	306.5±6.9[a,b]

Data are expressed as means±SD of seven animals.

Unit of Vit C is µg AA/g tissue

Unit of TBARS is µM MDA / hr / g tissue.

Unit of GSH is µmol/g of tissues.

[a]Significantly different from the control group (ANOVA/Duncan, $p < 0.0001$).

[b]Significantly different from the diabetic group (ANOVA/Duncan, $p < 0.001$).

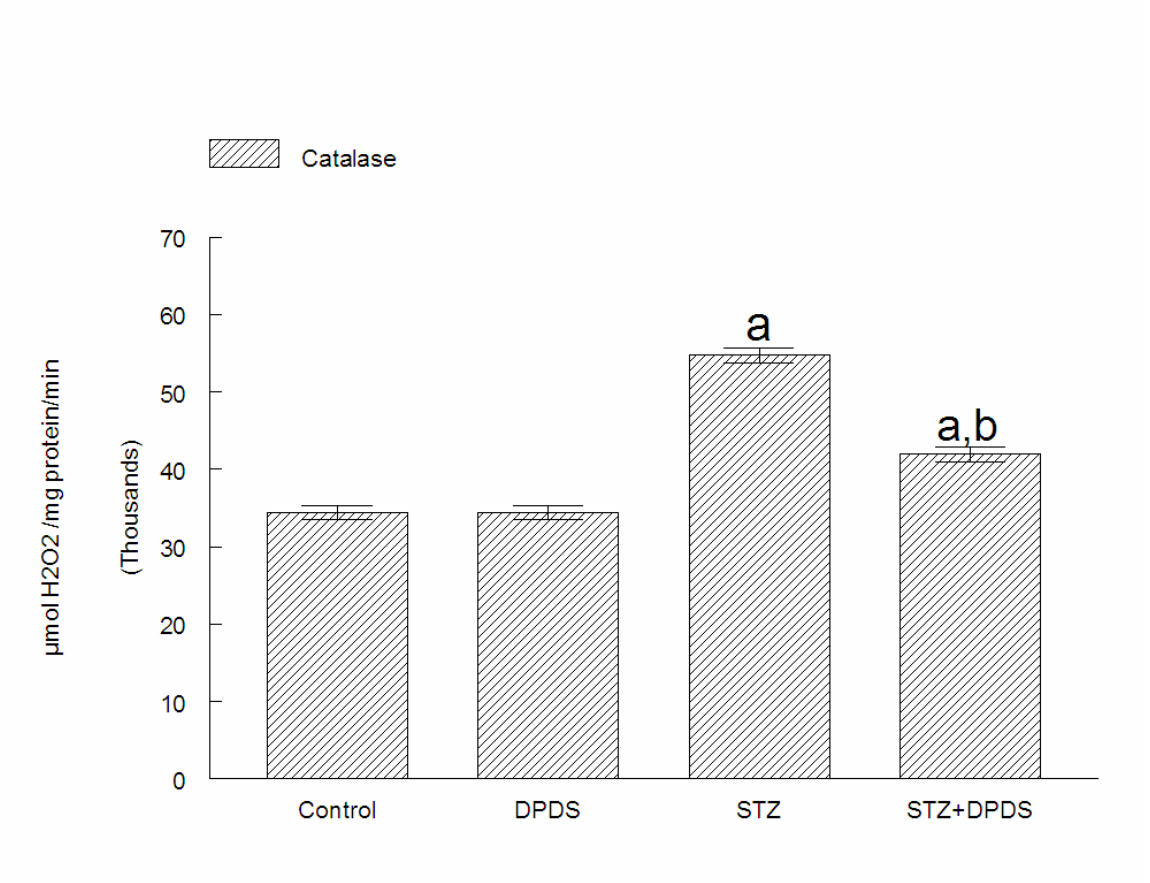

Figure 1: Cerebral Catalase activity (means±SD, n = 7) of STZ and DPDS treated rats. STZ (group 3) and STZ+DPDS (group 4) treated groups were significantly different from the control group (ANOVA/Duncan, $p < 0.005$). In addition group 4 is significantly different from group 3.

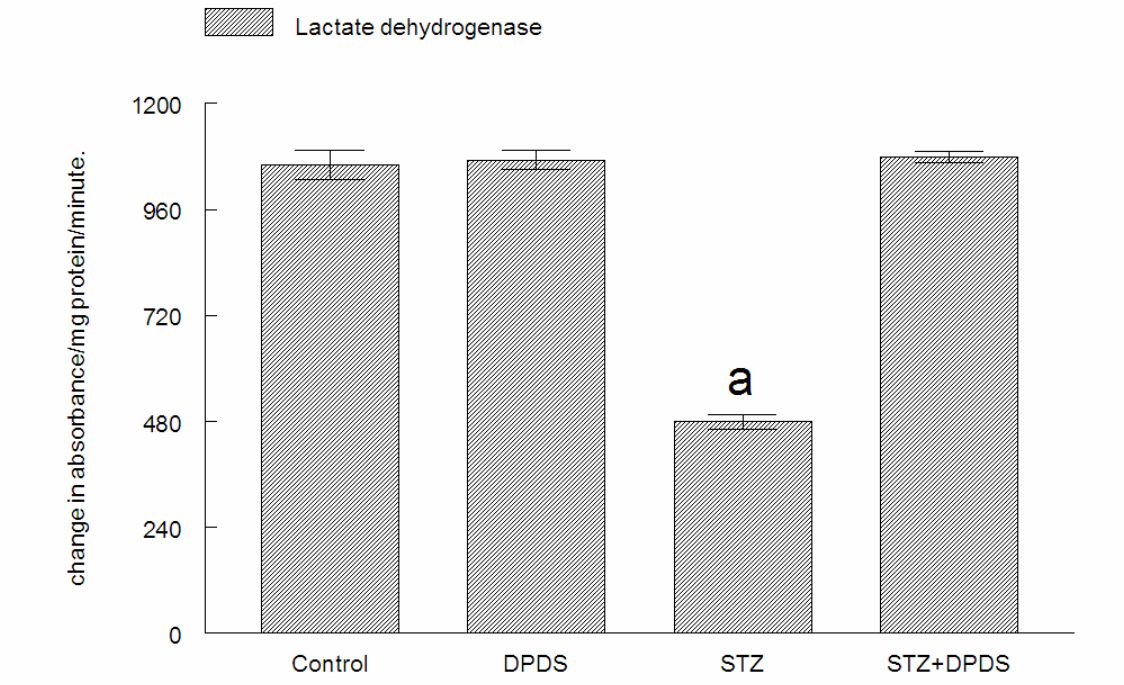

Figure 2: Cerebral LDH activity of STZ and DPDS treated rats. Data are expressed as mean±SD of seven animals. STZ treated rats (group 3) are significantly different from the control group (ANOVA/Duncan, $p < 0.005$).

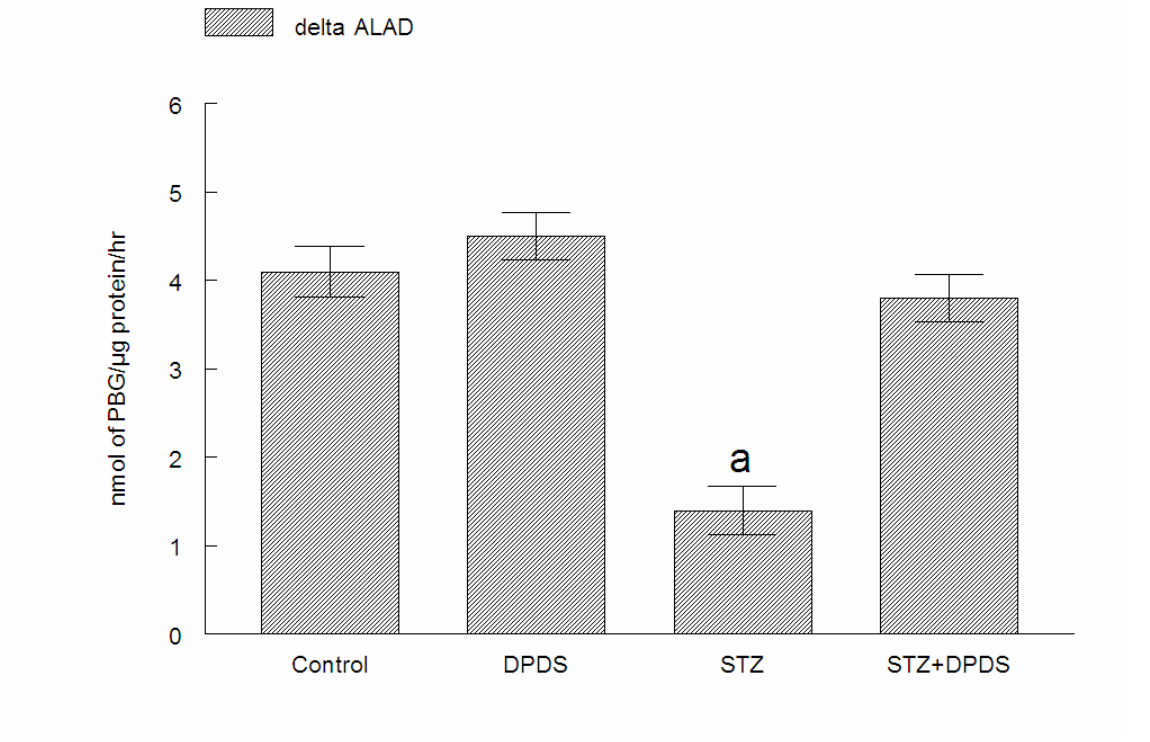

Figure 3: Cerebral ð-ALA-D activity of STZ and DPDS treated rats. Data are expressed as mean±SD of seven animals. STZ treated rats (group 3) are significantly different from the control group (ANOVA/Duncan, $p < 0.0005$).

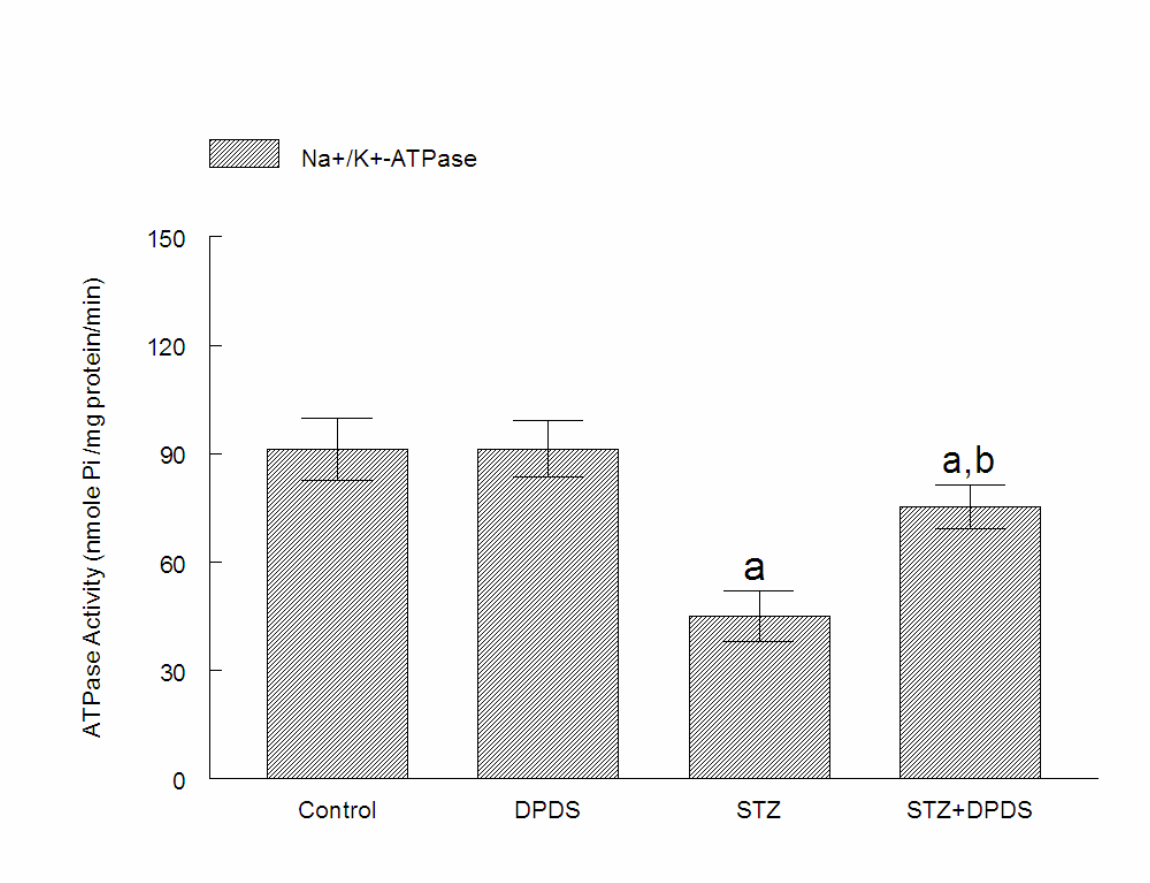

Figure 4: Activity of cerebral Na^+/K^+-ATPase in STZ and DPDS treated rats. Data are expressed as mean±SD of seven animals. STZ (group 3) and STZ+DPDS (group 4) treated groups were significantly different from the control group (ANOVA/Duncan, $p < 0.0005$). In addition group 4 is significantly different from group 3.

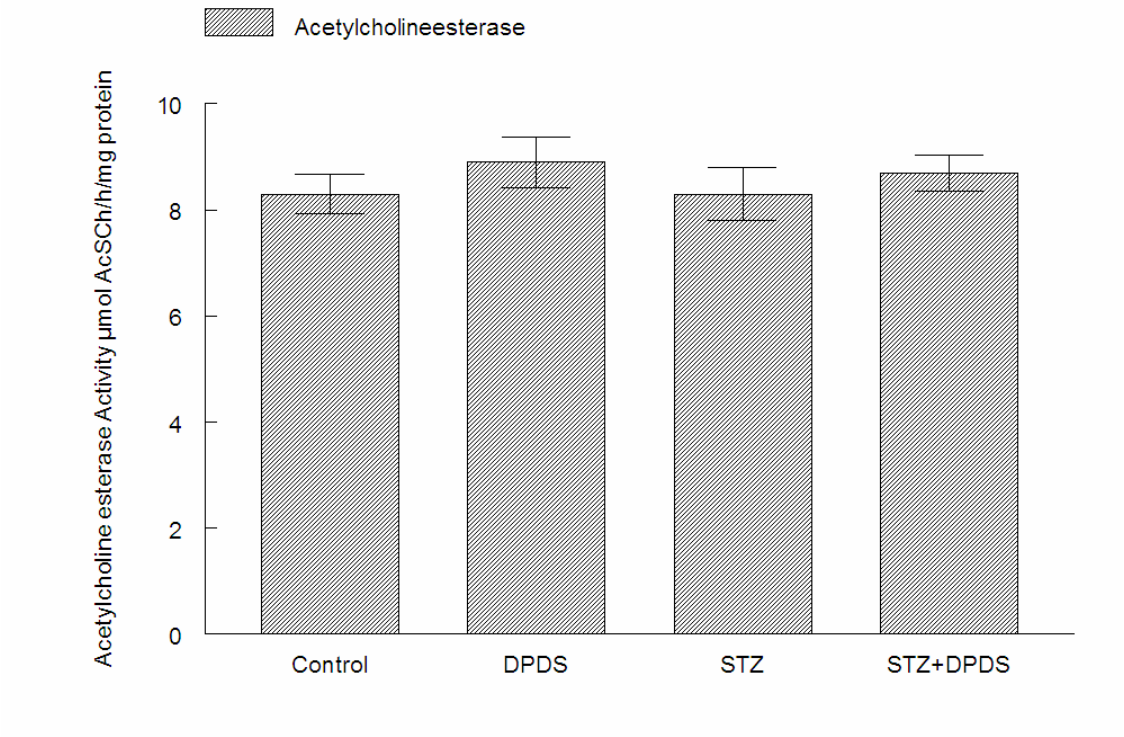

Figure 5: Activity of cerebral acetylcholineesterase in STZ and DPDS treated rats. Data are expressed as mean±SD of seven animals.

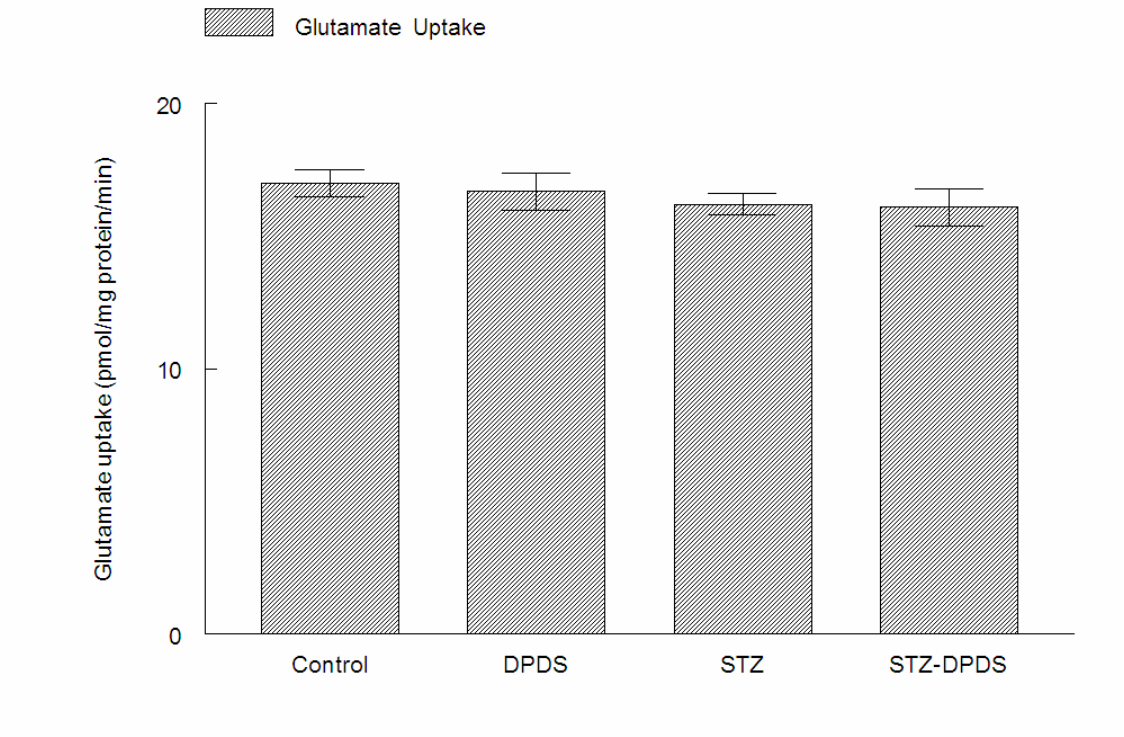

Figure 6: Glutamate uptake in the brain synaptosomes of STZ and DPDS treated rats. Data are expressed as mean±SD of seven animals.

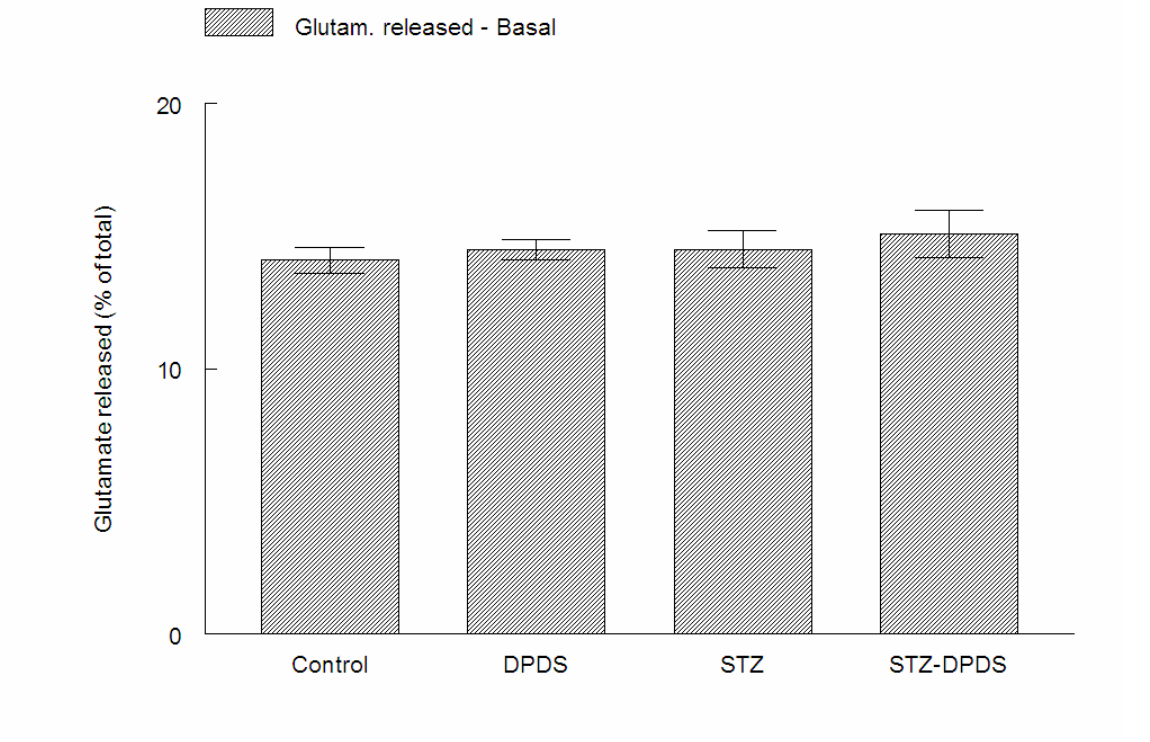

Figure 7: Basal glutamate released in cerebral synaptosomes of STZ and DPDS treated rats. Data are expressed as mean±SD of seven animals.

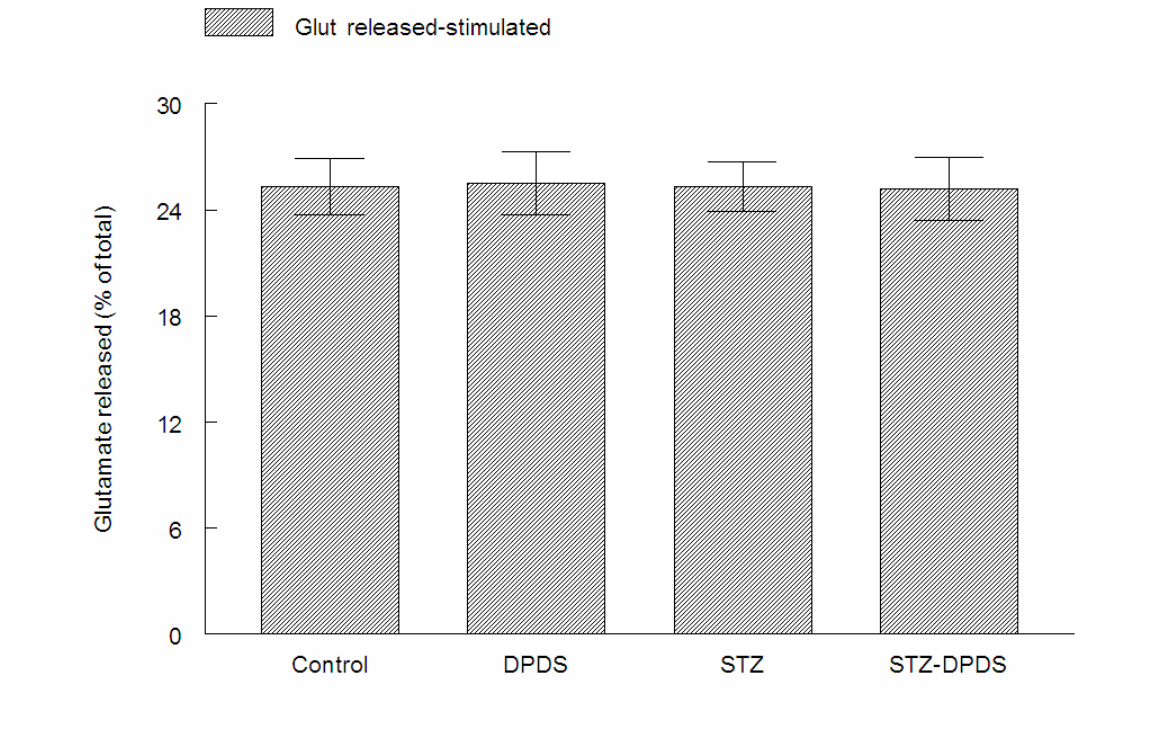

Figure 8: Stimulated glutamate released in cerebral synaptosomes of STZ and DPDS treated rats. Data are expressed as mean±SD of seven animals.

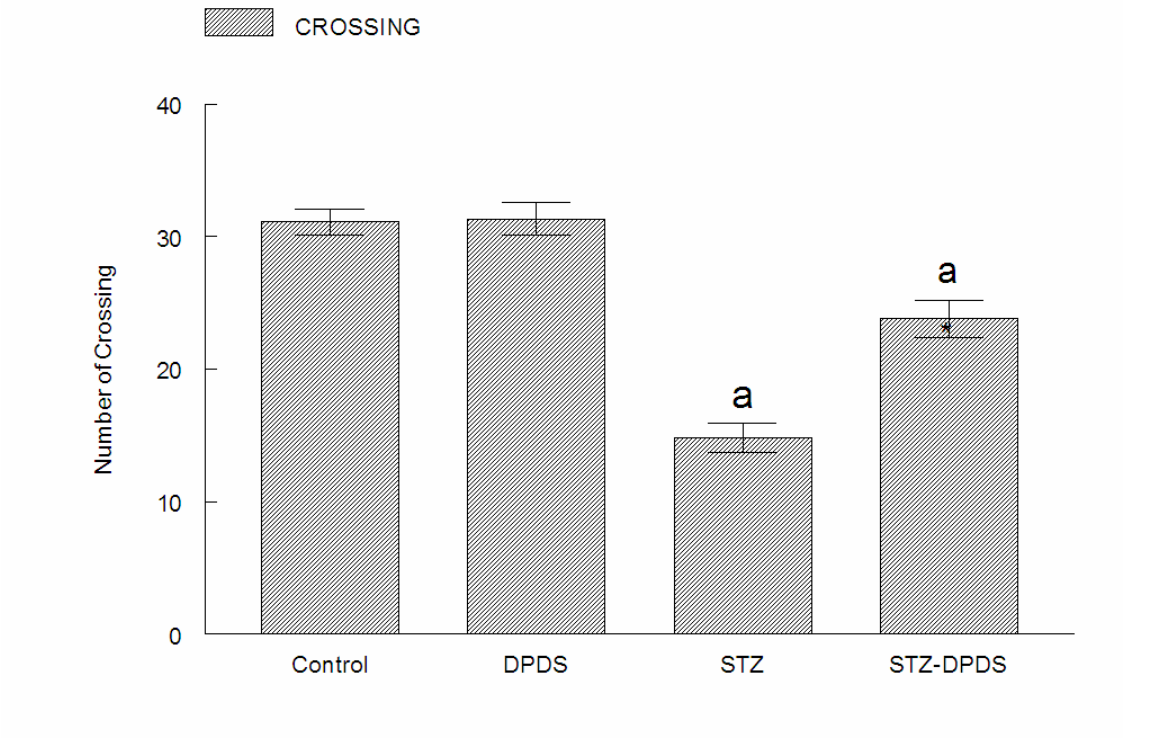

Figure 9: Number of crossings made by rats treated with STZ, DPDS or both in comparison with control. Data are expressed as mean±SD of seven animals. STZ (group 3) and STZ+DPDS (group 4) treated groups were significantly different from the control group (ANOVA/Duncan, $p < 0.005$). In addition group 4 is significantly different from group 3.

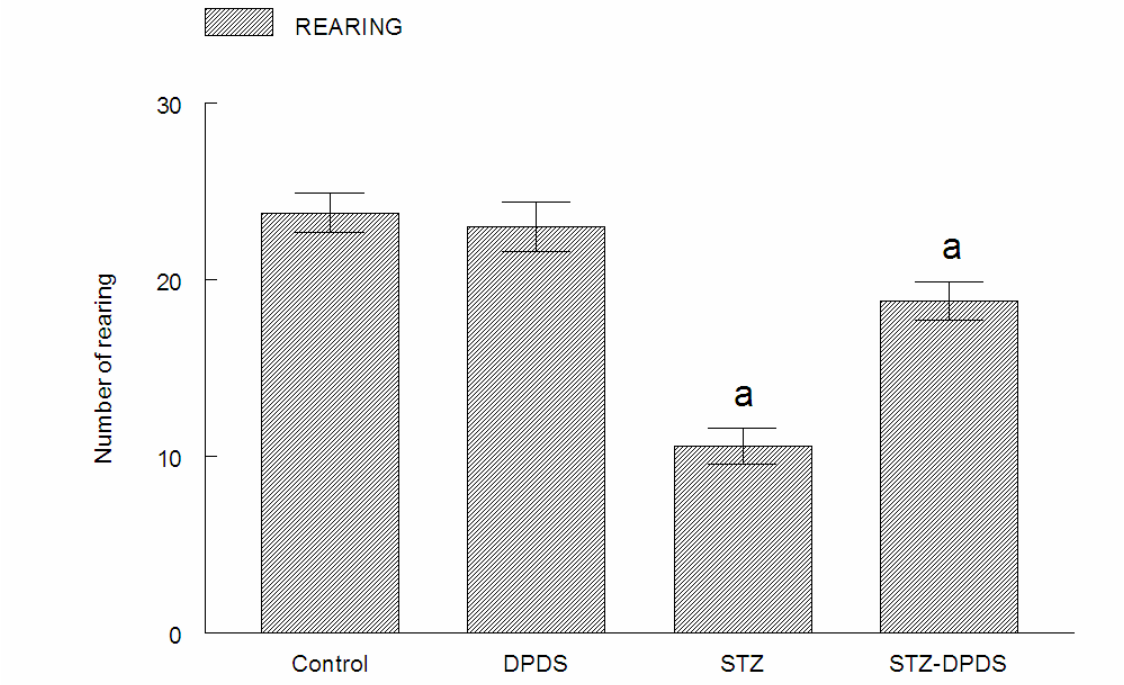

Figure 10: Number of rearing made by rats treated with STZ, DPDS or both in comparison with control. Data are expressed as mean±SD of seven animals. STZ (group 3) and STZ+DPDS (group 4) were significantly different from the control group (ANOVA/Duncan, $p < 0.0005$). In addition group 4 is significantly different from group 3.

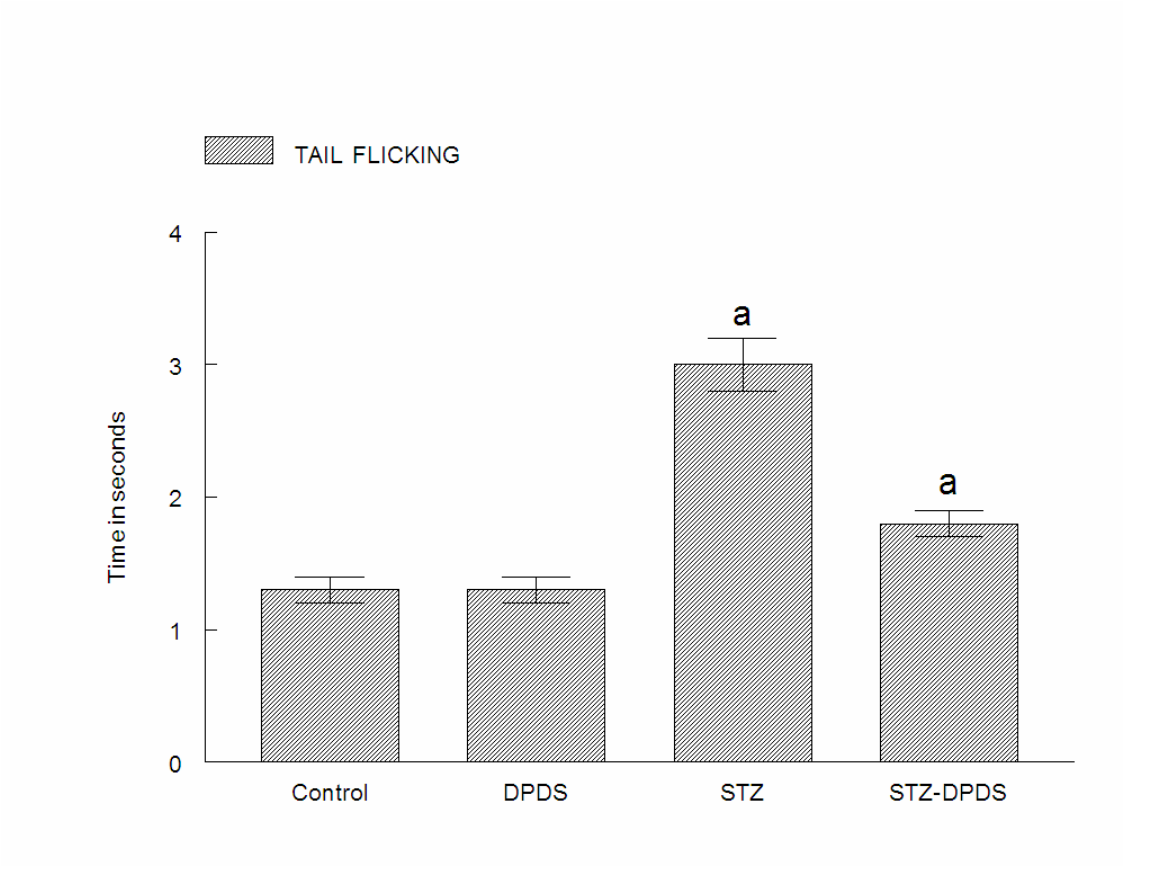

Figure 11: Time (in seconds) expended before rats treated with STZ, DPDS or both flicked their tails away from hot water at 55^0C. Data are expressed as mean±SD of seven animals. STZ (group 3) and STZ+DPDS (group 4) treated groups were significantly different from the control group (ANOVA/Duncan, $p < 0.0005$). In addition group 4 is significantly different from group 3.

4 General Discussion and Conclusion

The precise mechanisms of organoselenium toxicity are complex and are far from being completely understood. Although there are strong lines of evidence suggesting that selenium compounds interact with critical sulphydryl groups that are essential for the catalytic activities of enzymes, till date, the precise location of these critical thiols on proteins are yet to be elucidated. The present study has clearly demonstrated that for Na^+/K^+-ATPase, the critical thiol groups that are target of organodiselenide oxidation is located at the ATP binding site of the enzyme (Kade *et al.,* 2007). Likewise, organodiselenides evidently interact primarily with the sulphydryl groups at the NAD^+ binding site of lactate dehydrogenase. While the present study on lactate dehydrogenase is not all inclusive, hence it is possible that sites of pyruvate and lactate binding may also contain some critical thiols that interact with selenium compounds. Strong evidence for this speculation stem from the results obtained with cardiac isoforms of the enzyme, where in the absence of lactate and in the presence of NAD^+, the enzyme was still inhibited by diphenyl diselenide and dicholesteroyl diselenide, suggesting that both diorganyl diselenide may be interacting with groups at the lactate binding domain.

In addition, we equally observed from the present study that the non-selenium moieties of organodiselenides are critical for the pharmacological potency and toxicity of organochalcogens. In this regard, we observed that dicholesteroyl diselenide (DCDS) (a more voluminous compound than ATP) failed to significantly inhibit the activity of Na^+/K^+-ATPase, whereas it markedly inhibited the activity of lactate dehydrogenase, possibly because NAD^+ and DCDS may occupy same volume on the enzyme. However, diphenyl diselenide potently inhibited the activities of the two enzymes. Hence, we in conformity with other speculations (Meotti, *et al*., 2004), we observed that the smaller size

organodiselenides are promising pharmacologically, although they are apparently more toxic.

Although *in vitro* data do not always correlate with *in vivo* observations and this can be observed in the present study. Obviously DPDS is potentially toxic *in vitro* due to its inhibitory effect on all thiol containing enzymes. However, a contrast effect was observed when DPDS was administered at pharmacological dose of 3mg/kg bw to rats previously treated with STZ. In fact, DPDS not only relieved the inhibitory effect of STZ on thiols containing enzymes tested such as Na+/K+-ATPase, LDH and ð-ALA-D but also had a sparing effect on the levels of non protein thiol contents of the cells such as GSH. DPDS like other organoselenium compounds mimic glutathione peroxidase enzyme in the antioxidant action, and consequently use GSH in the reduction of hydrperoxides and peroxynitrites. Therefore, the present study on diabetes indicate that organoselenium compounds may be either interacting with GSH synthesis in vivo or may choose to act via another mechanism different from their general GPx mimics. In addition, the inhibitory effects of thiol containing enzymes by STZ which was relieved by DPDS treatment also suggest that this enzymes inhibition by STZ may involve a system that may not be related to thiol oxidation on the enzymes. This fact is borne from the fact that DPDS under in vitro conditions caused an inhibition of thiol enzymes possibly by oxidaizing their critical thiol groups. Hence we may speculate that although the precise mechanisms associated with these enzymes inhibition in diabetes models may possibly involve glycation of these proteins. In conclusion, the precise mechanisms that may be involved in STZ induced diabetes and the pharmacological action of organoselenium are yet to be fully understood. However the present study holds great promise that organoselenium compounds are first line candidates in the management of diabetic conditions.

5 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 pharmacopotency of these organoselenium compounds vis-a-vis these diseases are future research themes.

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