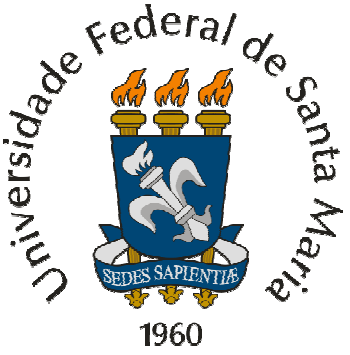

UFSM

Doctor of Philosophy (PhD) Thesis

Influence Of pH On the Reactivity of Organochalcogens with Thiols Of Biological Significance And Anti-Oxidant Potential In Rat's Tissue And Phospholipid Extracts

By

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Santa Maria, RS-Brazil

2009

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Dedicated To

My Family!

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For their kind and friendly support during this project.

Organization of the thesis

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

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

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

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

1.0 Summary

This study provides experimental data about the anti-oxidant activity of diphenyl diselenide, diphenyl ditelluride, a novel organoselenium compound and ebselen under various pH conditions. Iron is more soluble at lower pH values; therefore we hypothesized that decreasing the environmental pH would lead to increased iron-mediated lipid peroxidation. Because of the pH dependency of iron redox cycling, pH and iron need to be well controlled and for the reason we tested a number of pH values (from 7.4 to 5.4) to get a closer idea about the role of iron under various pathological conditions. Acidosis increased rate of lipid peroxidation in the absence Fe (II) in rat tissue homogenates especially at pH 5.4. This higher extent of lipid peroxidation can be explained by; the mobilized iron which may come from reserves where it is weakly bound. Addition of iron (Fe) chelator desferoxamine (DFO) to reaction medium completely inhibited the peroxidation processes at all studied pH values including acidic values (5.8-5.4). In the presence of Fe (II) acidosis also enhanced detrimental effect of Fe (II) especially at pH (6.4-5.4). All Tested compounds significantly protected lipid peroxidation at all studied pH values, except ebselen which offered only a small statistically non-significant protection. The highest anti-oxidant potency was observed for diphenyl ditelluride. These differences in potencies were explained by the mode of action of these compounds using their catalytic anti-oxidant cycles. We have also tested the thiol oxidase activity of diphenyl ditelluride as thiol oxidation by diphenyl ditelluride is a favorable reaction and may be responsible for alteration in regulatory or signaling pathways. We have measured rate constants for reactions of diphenyl ditelluride with cysteine, dimercaptosuccinic acid, glutathione and dithiothreitol in phosphate buffer. The relative reactivities of the different thiols with diphenyl ditelluride were independent of the pKa of the thiol group, such that at pH 7.4, cysteine AND dithiothreitol were the most reactive and low reactivity was observed with glutathione and dimercaptosuccinic acid. The reactivity of diphenyl ditelluride was not modified by change in pH. Rate of oxidation increased with increasing pH for all thiols except dimercaptosuccinic acid, where the rate of oxidation was faster at low pH. This study provides in-vitro evidence for acidosis induced oxidative stress and in rat tissues and potential anti-oxidant action of

Our observations will be of importance in our understanding of pathologies which are associated with low tissue pH. These studies confirm that organochalcogens are redox active within physiologically relevant potential range. The implication from these results for a biological system is that these compounds may react with thiols on the basis of their chemical reactivity. If it is selective, accessibility or other molecular features may be more important determinants. Furthermore other aspect that deserve investigation is to determine a possible relationship between thiol-peroxidase activity of these compounds with the capacity of catalyzing thiol/sulfide exchange, and how these two chemical properties of selenide/tellurides correlates with their toxicological and pharmacological effects.

Key words; Organochalcogens , pH, Anti-Oxidant Activity and Thiols

2.0 Introduction

2.1 Sources and reactions of reactive oxygen species (ROS)

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons. The presence of unpaired electrons usually confers a considerable degree of reactivity upon a free radical. Those radicals derived from oxygen represent the most important class of such species generated in living systems [1]. ROS can be produced from both endogenous and exogenous substances. Potential endogenous sources include mitochondria, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation [2]. Mitochondria have long been known to generate significant quantities of hydrogen peroxide. The hydrogen peroxide molecule does not contain an unpaired electron and thus is not a radical species. Under physiological conditions, the production of hydrogen peroxide is estimated to account for about 2% of the total oxygen uptake by the organism. However, it is difficult to detect the occurrence of the superoxide radical in intact mitochondria, most probably in consequence of the presence of high SOD activity therein. Generation of the superoxide radical by mitochondria was first reported more than three decades ago by Loschen et al. [3]. After the determination of the ratios of the mitochondrial generation of superoxide to that of hydrogen peroxide, the former was considered as the stoichiometric precursor for the latter. Ubisemiquinone has been proposed as the main reductant of oxygen in mitochondrial membranes [4]. Mitochondria generate approximately 2–3 nmol of superoxide/min per mg of protein, the ubiquitous presence of which indicates it to be the most important physiological source of this radical in living organisms [5]. Since mitochondria are the major site of free radical generation, they are highly enriched with antioxidants including GSH and enzymes, such as superoxide dismutase (SOD) and

glutathione peroxidase (GPx), which are present on both sides of their membranes in order to minimise oxidative stress in the organelle [6]. Superoxide radicals formed on both sides of mitochondrial inner membranes are efficiently detoxified initially to hydrogen peroxide and then to water by Cu, Zn-SOD (SOD1, localized in the intermembrane space) and Mn-SOD (SOD2, localized in the matrix). Besides mitochondria, there are other cellular sources of superoxide radical, for example Xanthine oxidase (XO), a highly versatile enzyme that is widely distributed among species (from bacteria to man) and within the various tissues of mammals [7]. Xanthine oxidase is an important source of oxygen-free radicals. It is a member of a group of enzymes known as molybdenum iron–sulphur flavin hydroxylases and catalyzes the hydroxylation of purines. In particular, XO catalyzes the reaction of hypoxanthine to xanthine and xanthine to uric acid. In both steps, molecular oxygen is reduced, forming the superoxide anion in the first step and hydrogen peroxide in the second [1]. Additional endogenous sources of cellular reactive oxygen species are neutrophils, eosinophils and macrophages. Activated macrophages initiate an increase in oxygen uptake that gives rise to a variety of reactive oxygen species, including superoxide anion, nitric oxide and hydrogen peroxide [8]. Cytochrome P450 has also been proposed as a source of reactive oxygen species. Through the induction of cytochrome P450 enzymes, the possibility for the production of reactive oxygen species, in particular, superoxide anion and hydrogen peroxide, emerges following the breakdown or uncoupling of the P450 catalytic cycle. In addition, microsomes and peroxisomes are sources of ROS. Microsomes are responsible for the 80% H_2O_2 concentration produced in vivo at hyperoxia sites [9]. Peroxisomes are known to produce H_2O_2, but not $O^{2}\bullet-$, under physiologic conditions. Although the liver is the primary organ where peroxisomal contribution to the overall H_2O_2 production is significant, other organs that contain peroxisomes are also exposed to these

H_2O_2- generating mechanisms. Peroxisomal oxidation of fatty acids has recently been recognised as a potentially important source of H_2O_2 production as a result of prolonged starvation. The release of the biologically active molecules such as cytokines and others, from activated Kupffer cells (the resident macrophage of the liver) has been implicated in hepatotoxicological and hepatocarcinogenic events. Recent results indicate that there is a close link between products released form activated Kupffer cells and the tumour promotion stage of the carcinogenesis process [10].

Reactive oxygen species can be produced by a host of exogenous processes. Environmental agents including non-genotoxic carcinogens can directly generate or indirectly induce reactive oxygen species in cells. The induction of oxidative stress and damage has been observed following exposure to various xenobiotics. These involve chlorinated compounds, metal (redox and non-redox) ions, radiation and barbiturates. For example 2-butoxyethanol is known to produce ROS indirectly, which causes cancer in mice [11].

2.2 Iron physico-chemistry in biological system

2.3 Iron-induced redox reactions,

Redox cycling is a characteristic of transition metals such as iron. Iron-catalyzed oxidative stress is believed to be the main mechanism involved in the pathogenesis of iron-induced cancer [12]. Reactive oxygen species (ROS), often under pathological conditions due to oxidative stress, have been shown to be associated with a wide variety of diseases, such as carcinogenesis, inflammation, radiation, and reperfusion injury [13]. In biological systems, it is often considered that ROS induced by iron originate from the interaction of iron with enzymatically and/or non-enzymatically generated superoxide ($O^{2}\bullet-$) (Haber–Weiss reaction) and/or hydrogen peroxide (H_2O_2) (Fenton reaction) [14,15]. However, measurements in liver cells have determined the steady state level of H_2O_2 to be

approximately 10−8M [16], and the steady state level of O_2 in vivo is about 10−5M [17]. Assuming that the rate constant for oxidation of substrate by "Fe^{2+} + O^2" chemistry (Fe^{2+} autoxidation) is similar to the Fenton reaction and that the oxidizable substrate concentration of a living system is about 1M, it has been estimated that the rate of oxidation of oxidizable substrate by "Fe^{2+} + O^2" could be as much as 108 faster than the rate of oxidation by the Fenton reaction [18]. These results suggest that "Fe^{2+} + O^2" chemistry is probably the most important route for free radical biology of iron. In fact, O_2•− and H_2O_2 may be produced directly from dissolved oxygen (O_2) in aqueous media in the Fe2+-mediated autoxidation reactions as follows:

Fe^{2+} + O2 → Fe^{3+} + $O^{2\bullet-}$ (1)

Fe^{2+} + $O^{2\bullet-}$ + $2H^+$ → Fe^{3+} + H_2O_2 (2)

Fe^{2+} + H_2O_2 → Fe^{3+} + OH^- + $OH^\bullet$ (3)

In comparison with Fenton/Haber–Weiss reactions where iron is catalytic or redox cycled, iron is consumed in iron autoxidation reactions. For example, oxidants produced by the interaction of Fe^{2+} and O^2 may be quenched by Fe^{2+} itself at the high concentrations as follows:

Fe^{2+} + $OH^\bullet$ → Fe^{3+} + OH_- (4)

According to the reactions (1)–(4), a self-quenching reaction can be written as follows:

$4Fe^{2+}$ + O_2 + $2H^+$ → $4Fe^{3+}$ + $2OH^-$ (5)

A total stoichiometry 4 Fe^{2+}:1 O_2 can be proposed based on the reaction (5), though this stoichiometry is greatly dependent upon the nature of the iron chelator used, and can differ markedly from the 4:1 [19,20].

It is known that the activation of oxygen by iron is subject to both kinetic and thermodynamic restraints, and therefore, reactions (1)–(3) as described are oversimplified.

For example, it has been suggested that oxidants other than the hydroxyl radical ($OH^{\bullet}$), such as ferryl or iron oxo, may also be generated [21, –22]. Because the conversion of H_2O_2 and O_2 into the more reactive $OH^{\bullet}$ radical requires the participation of Fe^{2+}, and DFO binds tightly only to Fe^{3+}, it has been recently shown that the combination of a Fe^{2+} chelator 2,2_-dipydyl with DFO had the most significant effect in preventing cells from iron-induced lipid peroxidation in human liver HepG2 cells [23].

2.4 pH dependency of iron-induced redox reactions

Chemically speaking, ferric ion (Fe^{3+}) is a weak oxidant, and ferrous ion (Fe^{2+}) is the form of iron that is capable of redox cycling. Oxidation of Fe^{2+} to Fe^{3+} resulting in ROS formation is greatly dependent upon the pH of the media. For example, the reaction half lives of Fe^{2+} at pH 3.5 and 7.0 were 1000 days and 8 min, respectively [24, 25]. In a pH buffered system under a constant partial pressure of oxygen, the Fe^{2+} oxidation proceeds at pH values greater than 4.5 with the kinetic relationship:

$-d[Fe^{2+}]/dt = k[Fe^{2+}][O_2][OH^-]2$

where k = 8.0 × 1013 l2/(mol2 atm min) at 25 ◦C. Increasing the pH into the alkaline region causes the precipitation of ferrous hydroxide, which causes the rate of oxidation to change from a homogenous to a heterogenous reaction and leads to a further increase in the rate [26]. At pH values below 3.5, the reactionproceeds at a rate independent of pH:

$-d[Fe^{2+}] / dt = k_[Fe^{2+}][O_2]$

where k_ = 1.0 × 10−7 atm−1 min−1 at 25 ◦C. Since Fe^{2+} oxidation is very slow at pH < 3.5, there is consequently not much cytotoxicity in the gastric system (pH 1–2) caused by oral uptake of a normal dose of ferrous sulfate. However, in lung medium in which the pH is usually greater than 7, oxidation of Fe^{2+} probably proceeds quickly and ROS resulting from the interaction of Fe^{2+} and O_2 may damage lung cells and cause cytotoxicity. This

suggests that the inhalation of iron may be more hazardous than the ingestion of iron [27]. In fact, pulmonary injury after aspiration of $FeSO_4$ has been reported in a patient showing acute bronchial damage and early histological change in the biopsy specimens after the exposure [28]. A delayed occurrence of bronchial stenosis after inhalation of iron has also been described [29, 30]. Because of the pH dependency of iron redox cycling, pH and iron need to be well controlled in cell experiments involving iron-mediated free radical oxidation. It was shown that acidic pH (<4.5) stabilized $FeSO_4$ in the coal dusts, whereas at high pH the conversion of reactive Fe2+ to Fe3+ was immediate [31]. Iron-catalyzed lipid peroxidation in K-562 cells was shown to be pH dependent, the lower the extracellular pH (decreasing from 7.5 to 5.5), the higher the free radical flux; the lower the pH, the greater the membrane permeability of iron [32]. In the phagolysosomes of cells where pH is around 5.5 [33], this pH environment seems to provide optimal conditions for maximal catalytic efficiency and solubility of iron [34, 35]. In comparison with normal tissue, human tumors have relative low pH levels [36]. These low levels in tumors may increase iron released from ferritin and lactoferrin and enhance oxidative damage as well.

2.5 Selenium (Se) and Tellurium (Te) Based Anti-Oxidants

Organoselenium compounds exhibit potent antioxidant activity mediated by their glutathione peroxidase (GPx) mimetic properties. In line with this, ebselen (pz 51; 2-phenyl-1,2-benzisoselenazol-3(2H)-one), a synthetic selenium-containing heterocycle has been used experimentally and clinically with success in a variety of situations where free radicals are involved. Diphenyl Diselenide (DPDS), the simplest diaryl diselenide, is particularly important as a potential antioxidant drug in view to the fact that it has been shown to be more active as a glutathione peroxidase mimic, less toxic to rodents than ebselen and has also low toxicity for non-rodent mammals after long term exposure [37].

Similarly, There has been a considerable interest in organotellurium compounds as potential antioxidants in living systems against several pro-oxidant agents, such as hydrogen peroxide, peroxynitrite, hydroxyl radicals and superoxide radical anion [38-43], since these compounds may mimic glutathione peroxidase activity (GPX) [44,45]. This property is thought to be due to oxidation of Te from the divalent to the tetravalent state. Besides, tellurides are promising antitumoral drugs and their chemoprotective effects can be related to their cytotoxic properties and to their ability to inhibit important enzymes necessary for the tumor growth [46-47]. These pharmacological properties are much more evident in organotellurium compounds than in selenium or sulfur analogues, making these compounds extremely attractive in medical therapies.

3.0 Objectives;

Working hypothesis,

1.0 We have tried to explore the possibilities if change in pH could modulate anti-oxidant of organochalcogens, as based on the redox potential and electrochemical studied on organochalcogens, we worked on the hypothesis that the formation of stables selenolate (Se^{-1}) and tellurate (Te^{-1}) ions can increase the reducing properties of these moieties on the organochalcogenides and hypothetically can increase their antioxidant properties. However, there is no data in the literature supporting this assumption.

2.0. Iron is more soluble at lower pH values; therefore we hypothesized that decreasing the pH of the reaction medium will lead to increased lipid peroxidation.

For the purpose,

The work was designed in this regard to get a different pH media (in-vitro) and study,

1. The effect of pH on Fe (II) mediated lipid peroxidation in rat's brain, kidney, liver homogenates and phospholipids extract from egg yolk by measuring thiobarbituric acid-reactive species (TBARS).

Similarly,

2. We have determined the influence of pH on protective effect of organoselenium and organotellurium compounds (in vitro) at different pH ranging from low (acidic) to physiological value in rat's brain, kidney, liver and phospholipids extract from egg yolk.

3.0 We have also studied the thiol oxidase activity of diphenyl diselenide and diphenyl ditelluride at various pH ranging from physiological value (7.4) to acidic values (5.4).

4.0 First Manuscript

pH-Dependent Fe (II) pathophysiology and protective effect of an organoselenium compound

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ABSTRACT

Influence of pH on the extent of lipid peroxidation and the anti-oxidant potential of an organoselenium compound is explored. Acidosis increased the rate of lipid peroxidation both in the absence and presence of Fe (II) in rat's brain, kidney and liver homogenate and phospholipids extract from egg yolk. The organoselenium compound significantly protected lipids from peroxidation, both in the absence and presence of Fe (II). Changing the pH of the reaction medium did not alter the anti-oxidant activity of the tested compound. This study provides in vitro evidence for acidosis-induced oxidative stress in brain, kidney, liver homogenate and phospholipids extract and the anti-oxidant action of the tested organoselenium compound.

1. Introduction

The role of iron in catalyzing oxygen-derived free radical production is well known, and there is evidence that free radicals may be a primary cause of cerebral damage during ischemia and post ischemic reperfusion [1]. The pH of tissue could modulate the ability of iron to participate in detrimental lipid peroxidation reactions. It has been suggested that metabolic changes induced by ischemia, such as acidosis [2] lead to intracellular iron delocalization [3] providing a source of iron in a form capable of catalyzing free radical production. Thus, the fall in intracellular pH that is associated with ischemia cannot only influence metabolic processes, but it can also potentate or act sinergically with oxidative stress, contributing to increased cellular injury. Iron is more soluble at lower pH values; therefore, we hypothesized that decreasing the pH of the reaction medium will lead to increased lipid peroxidation. For this purpose we have studied the effect of pH on Fe (II)-mediated lipid peroxidation in rat's brain, kidney, liver homogenates and phospholipids extract from egg yolk by measuring thiobarbituric acid-reactive species (TBARS).

Organoselenium compounds have been described to possess very interesting biological activities. Reports have shown that these selenium-containing organic compounds are generally more potent anti-oxidants than classical anti-oxidants and this fact serves as an impetus for an increased interest in the rational design of synthetic organoselenium compounds [4,5]. From a hypothetical point of view, the formation of stables selenolate (Se^{-1}) ions can increase the reducing properties of these moieties on the organochalcogenides and hypothetically can increase their anti-oxidant properties. However, there is no data in the literature supporting this assumption. Thus, to get a deeper insight into the potential use of an organoselenium compound 2-((1-(2-(2-(2-(1-(2-hydroxybenzylideneamino) ethyl) phenyl) diselanyl) phenyl) ethylimino) methyl) phenol (Compound A) (Fig. 1) as a possible pharmacological agent, we have determined for the first time the influence of pH on protective effect of Compound A in vitro at different pH ranging from low (acidic) to physiological values in rat's brain, kidney, liver and phospholipids extract from egg yolk.

2. Materials and methods

Compound A (Fig. 1) was synthesized according to literature methods [6,7] with little modifications. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that the compound obtained (with 99.9% purity) presented analytical and spectroscopic data in full agreement with their assigned structure. All other chemicals were purchased from standard suppliers.

Brain, kidney and liver was removed from adult male wistar rats, while phospholipids were extracted from eggs yolk by a solution 3:2 of hexane-isopropanol in the proposition of 1 g of egg yolk to 10 ml of this solution. The mixture was filtered and put in the rotary evaporator with the maximum temperature of 60 °C. Extract was weighed and dissolved in distilled water in the proposition of

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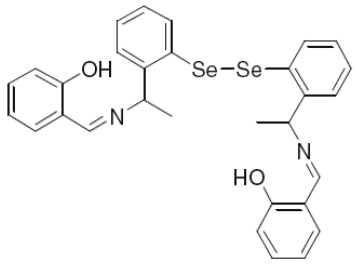

Fig. 1. Chemical structure of Compound A, i.e. 2-((1-(2-(2-(1-(2-hydroxybenzylideneamino) ethyl) phenyl) diselanyl) phenyl) ethylimino) methyl) phenol.

25 mg of extract to 10 ml of water which was used for (TBARS) assay. Lipid peroxidation was determined by measuring (TBARS) as described by Ohkawa et al. [8] with a slight modification that pH of the incubation medium was changed from 5.4 to 7.4 (i.e. 5.4, 5.8, 6.4. 6.8 and 7.4).

3. Results and discussion

Two-way analysis of variance (ANOVA) of Fe (II)-induced TBARS levels in rat's brain, kidney, liver and phospholipids extract from egg yolk revealed a significant main effect of pH and Fe (II) and also a significant Fe × pH interaction ($P < 0.05$). Indeed, lipid peroxidation in the absence of Fe (II) is enhanced upon a shift in the pH of the incubation solutions from physiological conditions (pH 7.4) to acidic ones (pH 5.4) as shown in (Fig. 2). Similarly, the amount of TBARS produced by incubation of homogenate and phospholipids extract with Fe (II) alone at pH 7.4 was lower. However, as the pH of the solution was decreased from 7.4 to 5.4, Fe (II)-dependent TBARS production markedly increased as shown in (Fig. 2).

The enhancement of pH dependent lipid peroxidation can be attributed to mobilized iron, which may come from reserves where it is weakly bound. It has been shown that the protein transferrin carries two iron ions, although only about one third of it is normally saturated with iron [9]. Transferrin loses its bound iron at acidic pH. The initial 10% of iron in saturated human transferrin is lost at a pH of 5.4 and the final 10% at a pH of 4.3 [10]. On the other hand, if transferrin is bound to its receptor, essentially all the iron is released at pH 5.6–6.0 [11]. The pH-dependent affinity of transferrin for iron decreases under acidic conditions leading to dissociation of iron from transferrin and other proteins like ferritin and lactoferrin [12]. Once mobilized, free iron likely binds non-specifically to a variety of small molecular moieties and augments the ordinarily small low molecular weight (LMW) non-protein-bound tissue pool. In cortical homogenates, striking increases in LMW iron are observed at pH 6.0 when pH is reduced from 7.0 by direct addition of lactic acid. Furthermore, brain from decapitated hyperglycemic rats shows elevated LMW iron relative to normoglycemic controls [13]. The mobilized iron Fe (II) can interact with enzymatically and/or non-enzymatically generated superoxide ($O_2^{\cdot -1}$) (Haber–Weiss reaction) and/or hydrogen peroxide (H_2O_2) (Fenton reaction) [14,15] producing reactive oxygen species. In fact, $O_2^{\cdot -1}$ and H_2O_2 may be produced directly from dissolved oxygen (O_2) in aqueous media in the Fe (II)-mediated basal/autooxidation reactions as follows:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot -1} \quad (1)$$

The dismutation of superoxide to hydrogen peroxide and oxygen has been shown to be faster at acidic pH [13]

$$HO_2^{\cdot} + O_2^{\cdot -1} + H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

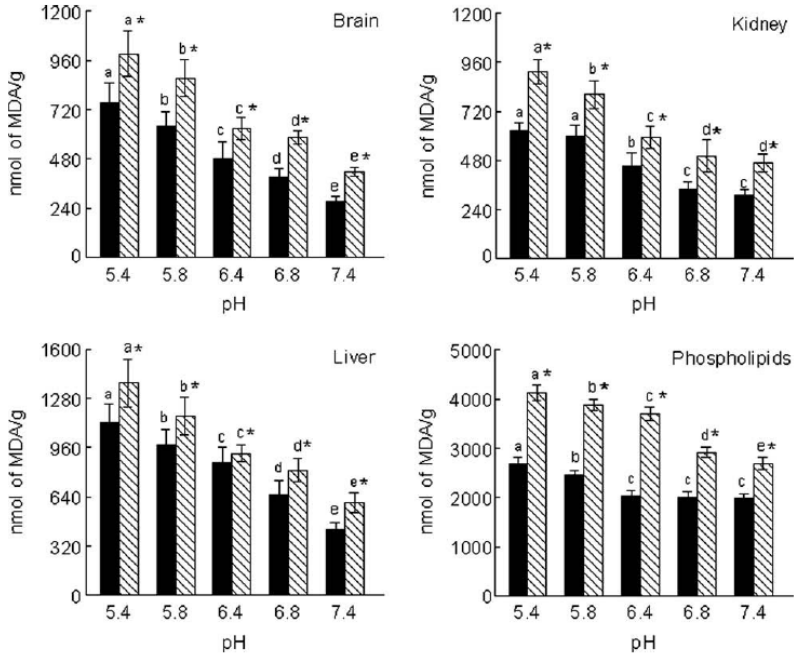

Fig. 2. Effect of pH on basal (shaded bar) or Fe (II)-induced (bar with lines) TBARS production in rat's brain, kidney, liver homogenate and phospholipids extract from egg yolk. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. ($n = 5–7$). Different letters shows significant difference from each pH group while asterisk shows significant main effect of Fe (II) at $P < 0.05$.

The H_2O_2 and superoxide produced in reactions above (1) and (2) may react together in a metal catalyzed (Haber–Weiss reaction) to produce the extremely reactive hydroxyl radical, which may then abstract hydrogen atoms from polyunsaturated fatty acids

$$Fe^{2+} + O_2^{\cdot -1} + 2H^+ \rightarrow Fe^{3+} + H_2O_2 \quad (3)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-1} + OH^{\cdot} \quad (4)$$

Ferrous ion (Fe (II)) is the form of iron that is capable of redox cycling. Oxidation of Fe (II) to Fe (III) resulting in ROS formation is greatly dependent upon the pH of the media. The possibility of reduction of Fe (III) to Fe (II) by interaction with $O_2^{\cdot -1}$ at the early phase of lipid peroxidation under acidic conditions, perhaps via an intermediate, perferryl iron [16], cannot be excluded

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot -1} \quad (5)$$

$$Fe^{3+} + O_2^{\cdot -1} \rightarrow [Fe^{3+} - O_2^{\cdot -1} \leftrightarrow Fe^{2+} - O_2] \leftrightarrow Fe^{2+} + O_2 \quad (6)$$

This pH environment and a series of chain reactions (1)–(6) seem to provide optimal conditions for maximal catalytic efficiency of iron. Thus, the acidic pH not only release iron from "safe" sites [11], but also potentiate the pro-oxidant effect of Fe (II), as we observed from significant increase in TBARS production at pH (6.8–5.4) in all tested homogenates and phospholipids extract (Fig. 2). The results are consistent with our previous observation that low pH indeed enhanced lipid peroxidation processes in brain and phospholipid extract [17,18].

Three way ANOVA of TBARS production, i.e. 6 pHs × 5 concentrations of Compound A × 2 (basal/iron) revealed a significant main effect of pH, Compound A and Fe (II). For the sake of clarity, data from the anti-oxidant effect of Compound A at different pH values and in the presence or absence of added Fe (II), which was analyzed by a three way ANOVA, was further analyzed by two-way ANOVA at each pH. Two-way ANOVA for TBARS production at all studied pH values revealed a significant main effect of Fe (II) and Compound A ($P < 0.05$ for all pH values) and also a significant Fe (II) × Compound A interaction ($P < 0.05$ for all pH values). It is possible to observe that Fe (II) increased TBARS production in all tested homogenates (Figs. 3–5), while, Compound A significantly reduced it.

Compound A also significantly reduced both basal and Fe (II)-induced TBARS at all studied pH values in phospholipids extract as shown in (Fig. 6). Data from the literature revealed the fact that Fe (II)-dependent TBARS production in phospholipids liposomes under acidic condition is not inhibited by the addition of SOD, catalase and ·OH scavengers (mannitol, sodium benzoate and dimethylthiourea) [19]. The lack of action of SOD, catalase and ·OH scavengers in Fe (II)-dependent TBARS production suggests that other species rather than $O_2^{\cdot -1}$, H_2O_2 and ·OH are involved in the initiation of Fe (II)-dependent lipid peroxidation. Several investigators have proposed some iron–oxygen complexes such as the ferryl ion [20], perferryl ion [16] and $Fe^{2+}—O_2^{\cdot}—Fe^{3+}$ complexes [21] for oxidizing species of Fe (II). A plausible mechanism by which Compound A is conferring protective action against Fe (II)-induced lipid peroxidation in these extracts is that Compound A could not only be working as a scavenger, but may also be interacting directly with Fe (II) or its oxidized forms. The reduced form of the compound was expected to be a good reducing agent, one that would be readily reactive towards ROS. However, contrary to our expectations, we did not find any alteration in the anti-oxidant activity with respect to pH of Compound A at all studied pH values.

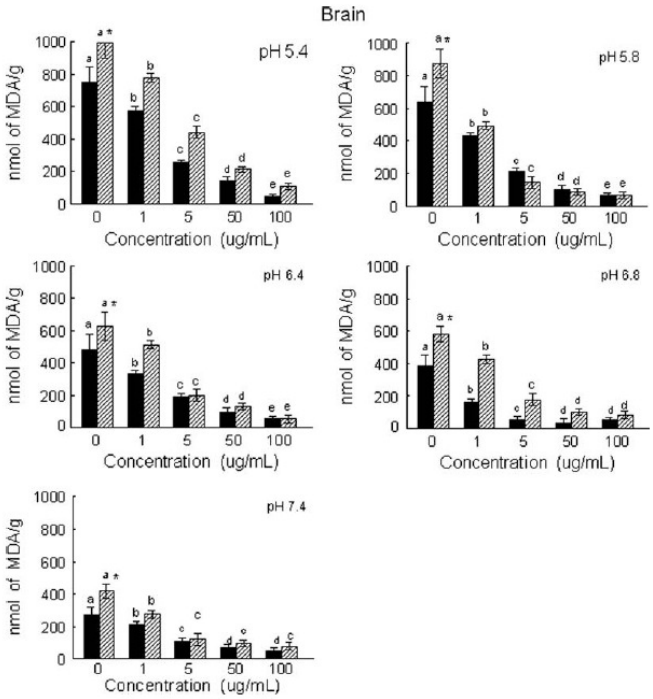

Fig. 3. Effect of Compound A on basal (shaded bar) or Fe (II)-induced (bar with lines) TBARS production in rat's brain homogenate at different pH. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. ($n = 5–7$). Different letters shows significant difference from basal group, while asterisk shows significant main effect of Fe (II) at $P < 0.05$.

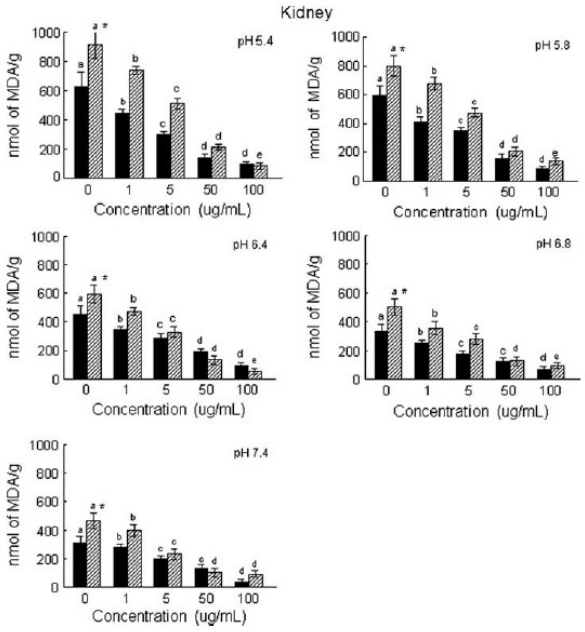

Fig. 4. Effect of Compound A on basal (shaded bar) or Fe (II)-induced (bar with lines) TBARS production in rat's kidney homogenate at different pH. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n = 5–7). Different letters shows significant difference from basal group, while asteric shows significant main effect of Fe (II) at $P < 0.05$.

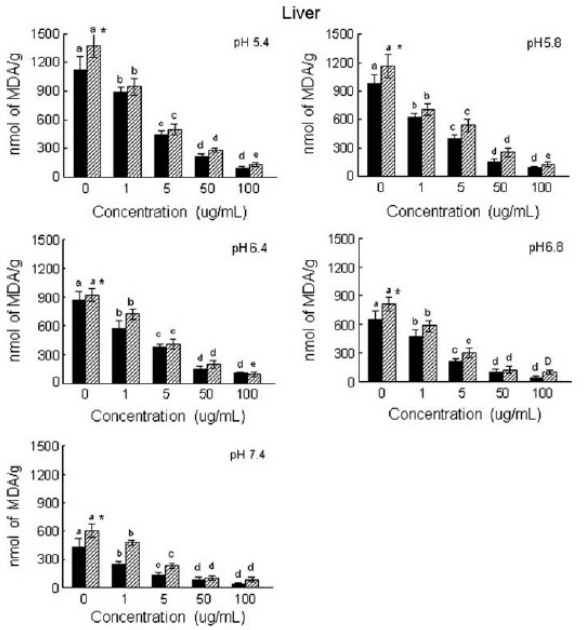

Fig. 5. Effect of Compound A on basal (shaded bar) or Fe (II)-induced (bar with lines) TBARS production in rat's liver homogenate at different pH. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n = 5–7). Different letters shows significant difference from basal group, while asteric shows significant main effect of Fe (II) at $P < 0.05$.

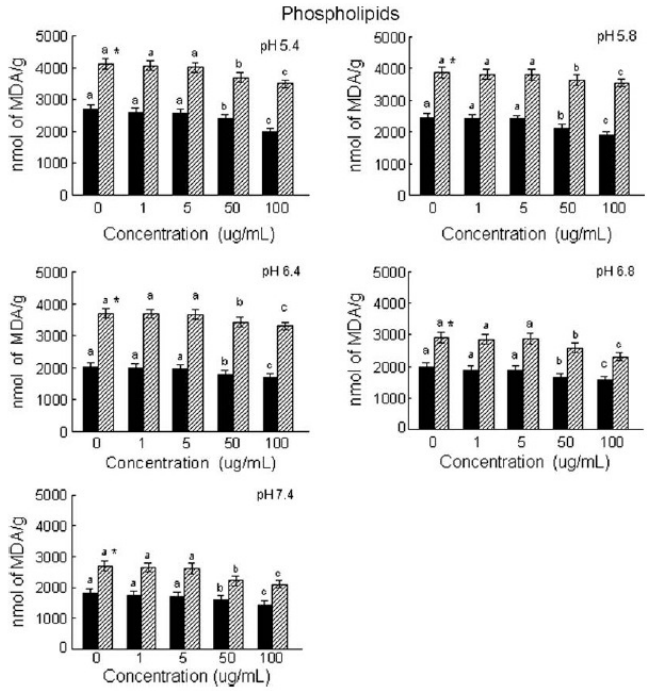

Fig. 6. Effect of Compound A on basal (shaded bar) or Fe (II)-induced (bar with lines) TBARS production in phospholipids extract from egg yolk at different pH. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. ($n = 5–7$). Different letters shows significant difference from basal group, while asteric shows significant main effect of Fe (II) at $P < 0.05$.

In the present work, we demonstrated for the first time the anti-oxidant effect of Compound A on Fe (II)-induced lipid peroxidation in rat's tissue homogenates and phospholipids extract in vitro, not only at physiological pH but also under acidic conditions. These results support the anti-oxidant potential of Compound A and a possible direct interaction with Fe (II) or its oxidized derivatives. Although the observation in the present study cannot be directly related to in vivo conditions, it seems that the results may give us a clue to understand the role of Fe (II) in the iron-mediated cell injury and/or diseases under acidic conditions and a possible anti-oxidant effect of Compound A in ischemic/reperfusion injury.

Acknowledgments

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4.1 2nd Manuscript

Influence of pH on the reactivity of diphenyl ditelluride with thiols and Anti-oxidant potential in rat brain

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Influence of pH on the reactivity of diphenyl ditelluride with thiols and anti-oxidant potential in rat brain

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ABSTRACT

Thiol oxidation by diphenyl ditelluride is a favorable reaction and may be responsible for alteration in regulatory or signaling pathways. We have measured rate constants for reactions of diphenyl ditelluride with cysteine, dimercaptosuccinic acid, glutathione and dithiothreitol in phosphate buffer. The relative reactivities of the different thiols with diphenyl ditelluride were independent of the pK_a of the thiol group, such that at pH 7.4, cysteine and dithiothreitol were the most reactive and low reactivity was observed with glutathione and dimercaptosuccinic acid. The reactivity of diphenyl ditelluride was not modified by change in pH. Rate of oxidation increased with increasing pH for all thiols except dimercaptosuccinic acid, where the rate of oxidation was faster at low pH. The lipid peroxidation product malonaldehyde (MDA) was measured in rat brain homogenate and phospholipids extract from egg yolk after incubation in phosphate buffer at various pHs ranging from 7.4 to 5.4. TBARS production increased when homogenates were incubated in the pH (5.4–6.8) medium both in the absence and presence of Fe(II). These data indicate that lipid peroxidation processes, mediated by iron, are enhanced with decreasing pH. The iron mobilization may come from reserves where it is weakly bound. Diphenyl ditelluride significantly protected TBARS production at all studied pH values in a concentration dependent manner in brain homogenate. This study provides in vitro evidence for acidosis induced oxidative stress and anti-oxidant action of diphenyl ditelluride.

1. Introduction

Tellurium compounds exhibit different biological activities. Compounds of the form R_2TeCl_n exhibit anthelmintic activity, while tellurite ions (TeO_3^{2-}) induce alterations of the erythrocyte membrane [1]. The latter activity is thiol dependent and probably involves intermediates with thiol-tellurium covalent bonds and various tellurium oxidation states. Organotellurium compounds can exhibit potent anti-oxidant activity mediated by their glutathione peroxidase (GPx) mimetic properties [2–7] and peroxinitrite scavenger abilities [8]. There is accumulative evidence suggesting that much of the biological activity of organotellurium compounds is directly related to their specific chemical interactions with endogenous thiols. Similarly, THE toxicity of organotellurium compounds is also mediated at least in part by their ability to react with thiols groups from biologically important molecules. Organochalcogens and in fact organotellurium compounds, including diphenyl ditelluride (DPDT), can inhibit thiol-containing enzymes, such as ALA-D [9–13], Na+, K+ ATPase, [14], catepsin B [15] and squalene monooxigenase [16]. The inhibition of squalene monooxigenase can contribute to demyelination and, consequently, to neurotoxic effects of organotellurium compounds [17,18].

To understand the mechanisms of interaction of tellurium compounds with biological systems at a molecular level, it is essential to expand our understanding of the tellurium-thiol chemistry. For this purpose we have studied the reactivity of 4 different thiols, i.e., glutathione (GSH), cysteine (CYS), dimercaptosuccinic acid (DMSA) and dithiothreitol (DTT) with DPDT and also reported influence of pH on the reactivity of DPDT with these biologically significant thiols.

It has been suggested that metabolic changes induced by ischemia, such as acidosis lead to intracellular iron delocalization providing a source of iron in a form capable of catalyzing free radical production [19–21]. Thus, the fall in intracellular pH that is associated with ischemia/acidosis can not only influence OVERALL metabolic processes, but also it can potentiate or act sinergically with oxidative stress contributing to increased cellular injury. Solubility of iron increases at lower pH values; therefore we hypothesized that decreasing the pH of the reaction medium will lead to increased lipid peroxidation. For the purpose influ-

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ence of pH (ranging from the physiological to the pathological values) on either basal or Fe(II) induced lipid peroxidation in rat's brain homogenate and phospholipids extracted from egg yolk was investigated.

From a hypothetical point of view the formation of stables tellurate (Te^{-1}) ions can increase the reducing properties of these moieties on the organochalcogenides and hypothetically can increase their anti-oxidant properties. However, there are no data in the literature supporting this assumption. Thus, to get a deeper insight about the potential use of organochalcogens as pharmacological agents, we also determined the influence of pH on the in vitro anti-oxidant activity of DPDT in brain homogenates and also in phospholipids extract. We have investigated whether DPDT could protect brain homogenates and phospholipids extract incubated with Fe(II) in vitro at different pH values.

2. Materials and methods

2.1. Chemicals

Dithiothreitol, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), glutathione, cystein, dimercaptosuccinic acid were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. DPDT synthesis and preparation

DPDT was synthesized according to literature method [22] and was dissolved in ethanol. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that the compound obtained presented analytical and spectroscopic data in full agreement with their assigned structure. The chemical purity of DPDT (99.9%) was determined by GC/HPLC. Solutions of DPDT were prepared in ethanol few minutes (5–10 min) before the experiments. The solution is stable for the period utilized in the experimental protocols performed in this study.

2.3. Preparation of buffers

All buffers were prepared at room temperature with constant ionic strength. Buffer solutions were maintained at 4 °C until the initiation of the experiment. Direct measurement of pH values in the tubes at higher temperature i.e. (37 °C) verified that actual pH values were typically within ±0.05.

2.4. The rate of thiol oxidation

Thiol oxidation was evaluated by measuring the disappearance of –SH groups according to the method of Ellman [23]. Incubation at 37 °C was initiated by adding specific concentration of GSH, CYS, DMSA and DTT to a medium containing 50 mM sodium phosphate buffer and a specific concentration of diphenyl ditelluride at different pH values. Aliquots of 60 μl were withdrawn at different interval of time and was used to determine the amount of –SH groups at 412 nm after reaction with 5,5′-dithio-bis(2-nitrobenzoic acid). Spectral measurements were performed by using a Hitashi 2001 spectrophotometer. Dependence of the rate of oxidation on pH was investigated in sodium phosphate buffer solutions at constant ionic strength. The pH values of the buffer solutions ranged from 5.4 to 7.8 i.e. (5.4, 5.8, 6.4, 6.8, 7.4 and 7.8) and were checked by using an Orion Research Digital pH/Millivolt Meter (Model 611). The reaction between DPDT and thiol was further studied in order to determine the reaction order. Its order in each thiol was determined by carrying out the reaction at a constant DPDT concentration (20 μM) and varying concentrations of thiols (0.1–2.0 mM). Progress of the reaction was followed by periodic titration of the residual free thiol with DTNB. In a second set of experiments, the reaction between thiol and DPDT was followed at a constant thiol concentration (1 mM) and varying DPDT concentrations (2.5–40 μM).

2.5. Animals

Adult male wistar rats from our own breeding colony (250–350 g) were maintained in an air-conditioned room (22–25 °C) under natural lighting conditions, with water and food (Guabi, RS, Brazil) ad libitum. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil.

2.6. Tissue preparation

Animals were anesthetized with ether and killed by decapitation. The brain was quickly removed, placed on ice, and homogenized within 10 min, in 10 mmol/l Tris/HCl buffer, pH 7.4 (in 10 volume). The homogenate was centrifuged at 4000 × *g* at 4 °C for 10 min to yield a low-speed supernatant fraction (S1) that was used immediately for TBARS assay [24].

2.7. Lipid peroxidation assay

Lipid peroxidation was determined by measuring thiobarbituric acid-reactive species (TBARS) as described by Ohkawa et al. [25] with minor modifications [24]. Tissue homogenate was prepared by homogenization as described above. Aliquots of the homogenate (100 μl) from brain was incubated for 60 min in a medium containing 10 mM sodium phosphate buffer of different pH ranging from 5.4 to 7.4 (pH was checked after the addition of brain supernatants both at the beginning and at the end of the incubation period) and in the presence of other reagents at concentrations indicated in the legends. The mixtures were incubated at 37 °C for 60 min. The reaction was stopped by addition of 0.5 ml of acetic acid buffer and lipid peroxidation products were measured by the addition of 0.5 ml of TBA 0.6% and 0.2 ml of SDS 8.1%. The color reaction was developed by incubating tubes in boiling water for 60 min. TBARS levels were measured at 532 nm using a standard curve of MDA. The values are expressed in nmol MDA/g of tissue. Where indicated, solutions of $FeSO_4$ were made just before use in distilled water.

2.8. Phospholipids extraction and TBARS production

Production of TBARS from phospholipid was determined using the method of Ohkawa et al. (1979) but with modifications. The egg yolk was weighed to 1 g and mixed with a solution of hexane–isoprapanol (3:2) and filtered. The solution was dried in a rotary evaporator at 60 °C. Then 0.2 g of the phospholipid was diluted to 10 ml with water, centrifuged and used as a homogenate. The remaining procedure was the same as that mentioned for the tissue except that the color reaction was carried out without SDS by adding 600 μl of TBA and 600 μl of acetic acid (pH 3.4) for 1 h. The tubes were cooled with tap water and 2 ml of n-butanol was finally added and the mixture centrifuged. The supernatant was taken and the absorbance was read at 532 nm in a spectrophotometer.

2.9. IC_{50} calculation

IC_{50} (concentration inhibiting 50% of lipid peroxidation) for lipid peroxidation was determined by the method of Dixon and Webb [26].

3. Statistical analysis

The results are expressed as the mean ± standard error (S.E.M.). Data were analyzed statistically by analysis of variance i.e. (Two and Three Way ANOVA), followed by univariate analysis and Duncan's multiple range test when appropriate. For the sake of clarity, data from the anti-oxidant effect of DPDT at different pH values and in the presence or absence of added Fe(II), which were analyzed by a three way ANOVA, were further analyzed by two-way ANOVA at each pH. Differences between groups were considered significant when $p < 0.05$.

Table 1
Second-order rate constants of reaction of the DPDT with various thiols at physiological pH (7.4).

Thiol	pK_a	k_2 (M^{-1} s^{-1})	
		DPDT	Ethanol
Cysteine (CYS)	8.3	1146 ± 49	959 ± 38
Dimercaptosuccinic acid (DMSA)	3.9	130 ± 17	86 ± 13
Dithiothreitol (DTT)	9.1	1114 ± 22	496 ± 12
Glutathione (GSH)	8.8	166 ± 8	128 ± 11

4. Results

4.1. Rates of reactions for thiols with DPDT

Oxidation of thiols by DPDT in phosphate buffer was followed by measuring the decrease in thiol concentration as a function of time. Loss of thiol was monitored by measuring the decrease in the absorbance at 412 nm due to the 5-thio-2-nitrobenzoate dianion (TNB) disulfide exchange reaction.

It is apparent from (Table 1) that the relative reactivities of the different thiols with diphenyl ditelluride were independent of the pK_a of the thiol group, such that at pH 7.4, cysteine and dithiothreitol were the most reactive and low reactivity was observed with glutathione and dimercaptosuccinic acid.

Rate constant for cysteine oxidation varied considerably as function of pH and on whether it was determined in the presence or absence of DPDT. Lowest reactivity was observed at pH 5.4; however the rate of oxidation increased with increasing pH both in the absence and presence of DPDT (Fig. 1).

Low reactivity was observed both in the case of DTT and GSH at low pH and both in the absence and presence of the tested compound i.e. (DPDT) as shown in (Fig. 1). The rate of oxidation increased with pH. Maximum oxidation was observed at pH 7.8 in the presence of DPDT; however, the rate of oxidation of DTT (Fig. 2) was much faster than GSH (Fig. 1).

It is apparent from (Fig. 1) that at low pH 5.4–5.8 the rate of reaction for DMSA was higher in the presence of DPDT. Increasing pH reduced the rate of reactions. In the absence of DPDT the rate

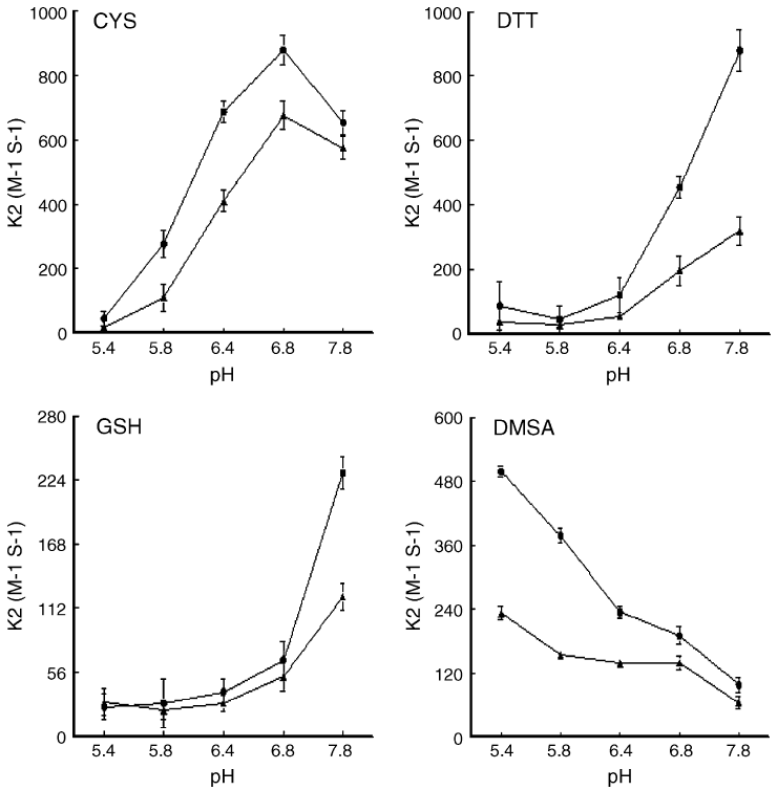

Fig. 1. pH-dependence of the second order rate constant of the reaction of DPDT (●) and ethanol (▲) with Cysteine, DTT, GSH and DMSA.

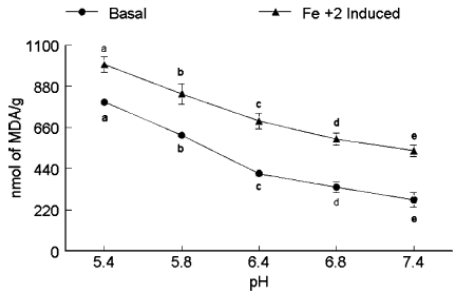

Fig. 2. Effect of pH on basal (●) and Fe(II)-induced (▲) (20 μM) TBARS production in supernatants of homogenates from brain. Low-speed supernatant (S1) from tissues were incubated for 60 min in sodium phosphate buffer of different pH. TBARS are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n = 5–7). Different letters show significant difference from each pH.

of reaction (for ethanol) was not modified in the range of 5.8–6.8, which gradually decreased at pH 7.8.

4.2. Effect of pH on basal and or Fe(II)-induced TBARS production in brain homogenate and phospholipid extract

Two-way ANOVA of Fe(II)-induced TBARS levels in brain homogenate (Fig. 2) and phospholipids extract (Fig. 3) revealed significant main effect of pH and Fe(II) and also a significant Fe × pH interaction ($p < 0.05$). Indeed, basal and Fe(II) induced TBARS production significantly increased by decreasing pH and this effect was more pronounced at pH 5.4 and 5.8 ($p < 0.05$).

4.3. Effect of DPDT on TBARS production in brain homogenate and phospholipids extract

Three way ANOVA of brain TBARS production revealed a significant pH × Fe(II) × DPDT interaction ($p < 0.05$). For the sake of clarity we have performed two-way analysis (Table 2) at each specific pH. Two-way ANOVA of brain TBARS productions at all studied pH values revealed a significant main effect of Fe(II), DPDT and significant Fe(II) × DPDT interaction ($p < 0.05$). Interaction was significant because the pro-oxidant effect of Fe(II) was evident either in the

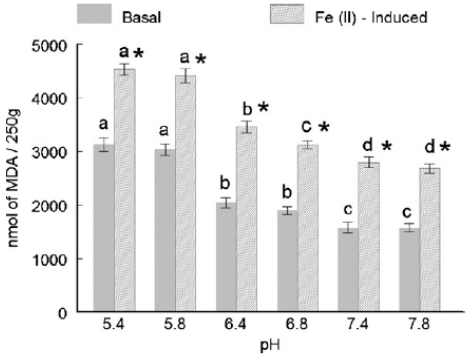

Fig. 3. Effect of pH on basal and Fe(II)-induced (20 μM) TBARS production in phospholipids extract from eggs. Low-speed supernatant (S1) from extract was incubated for 60 min in sodium phosphate buffer of different pH. TBARS are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n = 5–7). Different letters show significant difference from each pH. Different letters show significant difference from each pH while asterisks show significant effect of Fe(II).

Table 2
Effect of DPDT on basal or Fe(II)-induced (20 μM) TBARS production in supernatants of homogenates from brain of rats. Low-speed supernatant (S1) from tissues were incubated for 60 min in a medium containing 10 mM sodium phosphate buffer at different pH i.e. (5.4, 5.8, 6.4, 6.8, 7.4 and 7.8). The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n = 5–7). Different letters show main effect of DPDT while asterisks show main effect of Fe(II) at $p < 0.05$.

Treatment	pH				
	5.5	5.8	6.4	6.8	7.4
Basal (B)[a]	796 ± 23	618 ± 31	413 ± 31	341 ± 38	274 ± 21
B + 1 μM[a]	763 ± 19	584 ± 24	376 ± 39	311 ± 21	241 ± 25
B + 1.2 μM[b]	576 ± 15	410 ± 42	234 ± 22	243 ± 23	161 ± 19
B + 1.4 μM[b]	296 ± 21	242 ± 32	170 ± 18	159 ± 11	131 ± 13
B + 1.6 μM[b]	131 ± 17	113 ± 11	97 ± 9	84 ± 7	39 ± 12
Fe(II)-induced[a*]	996 ± 29	839 ± 53	694 ± 41	599 ± 42	536 ± 34
Fe(II) + 1 μM[a*]	939 ± 21	784 ± 31	647 ± 28	545 ± 26	503 ± 25
Fe(II) + 1.2 μM[b*]	676 ± 16	513 ± 23	428 ± 31	467 ± 31	341 ± 19
Fe(II) + 1.4 μM[b]	318 ± 32	217 ± 17	196 ± 23	284 ± 23	213 ± 17
Fe(II) + 1.6 μM[b]	127 ± 11	99 ± 11	67 ± 16	113 ± 11	44 ± 7

absence or in the presence of 1–1.2 μM of DPDT It is possible to observe that Fe(II) increased TBARS production while DPDT reduced it in a concentration dependant manner.

Two-way ANOVA of phospholipids TBARS productions at all studied pH values revealed only a significant main effect of Fe(II) ($p < 0.05$). DPDT did not offer protection against basal and Fe(II) induced TBARS production as shown in (Table 3)

5. Discussion

The calculated rate constants, extracted from the data and the corresponding graph showed 1st order kinetics in DPDT and overall 2nd order kinetics were observed for the reactions. The rate constants for CYS, DTT, GSH and DMSA are given in Table 1. It is evident from Table 1 that cysteine has higher rate constant as compared to GSH and DTT at physiological pH (7.4). The difference in the rates of oxidation both in the absence and presence of DPDT can be explained by the fact that both the extent of ionization of a thiol and the intrinsic nucleophilicity of the corresponding thiolate anion determine the overall reactivity of thiols in this type of reactions. It has been suggested that the lower the pK_a of a thiol the lower the nucleophilicity of the thiolate, but the higher the relative concentration of thiolate [27,28]. When the effect of the pH on the reactivity of thiols in these reactions were studied, it was found that, of the two opposite effects of the pK_a on the reactivity, the effect of the pK_a on the fraction of thiol that is deprotonated contributes more, if pH < pK_a [29]. This means that at physiological pH the CYS with the lower pK_a would be the better nucleophile than GSH and DTT in our experimental set-up.

We have also observed that GSH has lower rate constant than DTT at pH 7.4, both in the absence and presence of DPDT (Table 1). This difference can be explained by the fact that GSH is sterically more hindered than DTT which renders its rate of oxidation. Secondly, the close spatial proximity of two thiol groups in DTT not only facilitates auto-oxidation, but also promotes rate of oxidation in the presence of DPDT. Data presented here provide direct support to our earlier observation that ð-ALA-D (a sulfhydryl containing enzyme) from plants, in marked contrast to the enzyme from rats, was not inhibited by the diphenyl diselenide [10]. Infact plant enzyme has no cysteinyl residues in close proximity as observed in the active site of the mammalian enzyme. Similarly, our group has also observed that DTT is a better substrate than cysteine or GSH for the oxidation catalyzed by diorganoyl diselenides and diorganoyl tellurides [10]. The lower rate constants of DMSA can be attributed to extreme steric hindrance by carboxylic groups and low nucleophilicity of these thiols.

Table 3
Effect of DPDT on basal or Fe(II)-induced (20 μM) TBARS production in phospholipids extract from egg yolk. Low-speed supernatant (S1) from tissues were incubated for 60 min in a medium containing 10 mM sodium phosphate buffer at different pH. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n = 5–7). Asterisks show main effect of Fe(II) at $p < 0.05$.

Treatment	5.4	5.8	6.4	6.8	7.4
Basal (B)	3124 ± 134	3068 ± 122	2045 ± 111	1899 ± 97	1578 ± 134
B + 1 μM	3091 ± 141	3111 ± 111	2098 ± 123	1923 ± 111	1567 ± 98
B + 5 μM	3111 ± 101	3046 ± 127	2047 ± 134	1895 ± 98	1557 ± 85
B + 10 μM	3099 ± 79	3020 ± 131	2011 ± 122	1832 ± 76	1539 ± 111
B + 50 μM	3054 ± 121	2978 ± 143	1987 ± 134	1798 ± 111	1499 ± 151
Fe(II)-induced*	4531 ± 143	4213 ± 134	3456 ± 145	3122 ± 129	2789 ± 98
Fe(II) + 1 μM*	4553 ± 139	4256 ± 124	3441 ± 127	3111 ± 99	2754 ± 101
Fe(II) + 5 μM*	4532 ± 111	4239 ± 129	3434 ± 121	3102 ± 149	2739 ± 101
Fe(II) + 10 μM*	4511 ± 100	4209 ± 131	3412 ± 154	3123 ± 127	2709 ± 133
Fe(II) + 50 μM*	4494 ± 112	4184 ± 142	3386 ± 131	3098 ± 141	2688 ± 111

The variation of k_2 (expressed in terms of total thiol concentration) with respect to pH was studied to evaluate the contributions of the conjugate base, thiolate, produced in the equilibrium. Five to seven (5–7) runs were performed over the pH ranges 5.4–7.8 both (a) at constant thiol concentration and varying DPDT concentration and (b) constant DPDT and varying thiol concentration. The effect of pH on rate of reaction for CYS, DTT and GSH (Fig. 1) revealed that these thiols are more reactive when ionized and which is observable by gradual increase of rate constant with increasing pH both in the absence and presence of DPDT. This indicates that the deprotonated form of these thiols is the active species. Our study further revealed that the apparent rate constants for the reaction of DPDT with DMSA was higher at low pH as shown in (Fig. 1). Whereas, in the pH range investigated, the corresponding k_2 for other thiols increased with increasing pH. A possible explanation for the observed differences in pH-dependence between DMSA and these thiols could not be that the thiolate anion form is the actual substrate for DPDT. This is underlined by the similar pH profiles of DPDT reduction by other thiols. The question of higher reactivity at lower pH for carboxylic acid containing molecules may be related to a decrease in the reactivity of the negative charges at lower pH values i.e., the steric hindrance of the charges diminish as pH falls.

To predict the effect of acidic thiols on the pH of the reaction buffer, we considered a solution system, in which the acidic thiol (HS-R-COOH), pK_a of (COOH) ~3.5) – acting as a weak acid – is added to a buffer made from the conjugate acid–base pair, $H_2PO_4^-/HPO_4^{2-}$. Therefore, an equation for pH calculation, based on the principle of ionic equilibria, can be derived [30].

$$x^4 + (K_1 + K_2 + C_{b1})x^3 + [K_1(K_2 - C_{a1}) + K_2(C_{b1} - C_{a2}) - K_w]x^2 - [K_1K_2(C_{a1} + C_{a2}) + K_w(K_1 + K_2)]x - K_1K_2K_w = 0, \quad (1)$$

where x represents $[H^+]$; C_{a1} and C_{b1} are the concentrations for the conjugate acid–base pair and C_{a2}, the concentration of an added thiol; K_1 (=10^{-72}) and K_2 (=$10^{-3.5}$) are the acidity constants for $H_2PO_4^-$ and the acidic thiol (HS-R-COOH), respectively; and K_w is the dissociation constant of water. The pH value upon the addition of acidic thiols can be estimated by solving Eq. (1) for x. All acidic thiols exhibited pH-titration effect. The pH of phosphate buffer solution was reduced by a value of ~0.35–0.45 when the thiol concentration reached 2 mM. The observed pH changes can be predicted by Eq. (1), based on the assumption that the thiol carboxylates (HS—R—COOH) acted as weak acids in a buffer solution that contained a single conjugate acid–base pair ($H_2PO_4^-/HPO_4^{2-}$). The prediction was in general in agreement with the experimental data, with slight systematic overestimations of the pH value at lower thiol concentrations region (data not shown).

What are the implications of the present study, which mainly deals with the physio-chemical aspect of DPDT-Thiol reaction? One immediately following question would be: can the observed phenomena of pH, and therefore changes in rate of oxidation/reduction occur in biological systems or, specifically, in cells? The estimation in literature put the buffer capacity for whole blood in the range of 25–39 mM/pH, and for plasma (mainly bicarbonate buffer), 3 mM/pH. In addition, the buffer capacities for the cellular subcompartments—endoreticulum (ER), golgi complex, and endosome—were found to be between 6 and 50 mM/pH. Since thiol concentrations determined in cellular organelles are in the range of few micro molar to 11 mM, and the pH determined in different cellular compartments in the range of ℘ 5.0 to 7.5 [31,32] or even lower in tissues like stomach and urinary bladder., it is conceivable that DPDT–Thiol interaction profiles might be different in various organelles.

5.1. Effect of pH on basal and Fe(II)-induced production in brain homogenates and phospholipids extract

There are numerous pathologies that have associated oxidative stress as well as low tissue-pH values. The extracellular pH in human tumors can vary from 7.68 to 5.85. During global ischemia the extracellular space begins to go acid within 20 s of beginning anoxia or global ischemia. The magnitude of the fall in intracellular pH during global ischemia ranges from ~7.0 to 6.0 [33]. Similarly, in focal ischemia, it has been estimated that at the core, where blood flow was reduced to 25% at 10 min and to 10% by 3 and 4 h, pH_i fell to 6.6, 6.2, and 6.1 at those times [34]. In the penumbra, where flow was reduced to 40%, there was no significant pH_i drop at 3 h and a slight drop (to pH 6.75) at 4 h. Several groups have noted multiple pH profiles during ischemia using ^{31}P NMR, but pH values in relevant compartments have not been accurately determined. In one study, where the pH_i fall was measured in neurons with microelectrodes, pH_i fell to 6.3 in both CA1 (vulnerable) and CA3 (ischemia-resistant) cells [35].

These lower pH environments could not only influence metabolic processes, but they may also affect the detrimental oxidative processes in the tissue. The role of iron in catalyzing oxygen-derived free radical production is well known, and there is evidence that free radicals may be a primary cause of tissue damage during ischemia and post ischemic reperfusion. The pH of tissue could modulate the ability of iron to participate in detrimental lipid peroxidation reactions. It has been suggested that metabolic changes induced by ischemia lead to intracellular iron delocalization [36]. The following biologically compatible characteristics have compelled us to use iron as pro-oxidant. Iron is (1) exchangeable and chelatable in different pathophysiological conditions; (2) easily bioavailable for uptake by ferritin, heme and transferrin (3) metabolically and catalytically reactive in oxidant formation. In our study we have measured the pro-oxidant effect of iron at different pHs ranging from physiological to acidic ones i.e. 7.4–5.4, to get an insight about role of iron at different pathophysiological conditions.

Aliquots of brain homogenate (100 μl) were incubated for 60 min in a medium contains 10 mM sodium phosphate buffer, of different pH ranging from 5.4 to 7.4. The rate of peroxidation exhibits a dramatic increase as the pH is decreased from 7.4 to 5.4 as shown in Fig. 2. This pH dependency of TBARS production can be explained by the fact, firstly, that the protein transferrin carries two iron ions, although it is normally only about one third saturated with iron [37]. Transferrin loses its bound iron at acidic pH. The initial 10% of iron in saturated human transferrin is lost at a pH of 5.4 and the final 10% at a pH of 4.3 [37]. Whereas if transferrin is bound to its receptor, essentially all the iron is released at pH 5.6–6.0 [21]. The pH-dependent affinity of transferrin for iron decreases under acidic conditions leading to dissociation of iron from transferrin and other proteins like ferritin and lactoferrin [38]. Secondly, nitric oxide can interact with superoxide anions in the presence of [H^+] to yield peroxynitrite, which decomposes to hydroxyl radical and in turn enhances rate of lipid peroxidation. Indeed, transferrin has been shown to release bound iron during in vitro acidotic conditions, particularly as pH falls to <6.0 and brain cortical homogenates media release iron under acidic conditions [20]. Furthermore, acidosis increases TBARS in anoxic brain slices [19]. The data presented here provide direct evidence for enhanced lipid peroxidation in brain homogenate under acidic conditions.

Similarly, the results obtained revealed that lipid peroxidation in phospholipids extract in the absence of Fe(II) is enhanced upon a shift in the pH of the incubation solutions from physiological conditions (pH 7.4) to acidic ones (pH 5.4) as shown in (Fig. 3). Similarly, the amount of TBARS produced by incubation of phospholipids extract with Fe(II) alone at pH 7.4 was lower. However, as the pH of the solution was decreased from 7.4 to 5.4, Fe(II) dependent TBARS production markedly increased (Fig. 3). These results strongly suggest that the ability of Fe(II) to initiate lipid peroxidation is strengthened by increasing [H^+] and are consistent with our findings that low pH exacerbates iron-mediated lipid peroxidation processes in rat brain homogenate.

Data from our laboratory showed that DPDT can protect tissue homogenates against lipid peroxidation induced by various pro-oxidants [7 and 39]. However, there are no studies on the anti-oxidant activity of this compound at different pH values. DPDT significantly reduced both basal and Fe(II) induced TBARS at all studied pH values in a concentration dependent manner in brain homogenate as show in Table 2. The anti-oxidant potency can be explained by the fact that organotellurium compounds are readily oxidized from the divalent to the tetravalent state. This property makes them attractive as scavengers of reactive oxidizing agents such as hydrogen peroxide, hypochlorite, and peroxyl radicals, and as inhibitors of lipid peroxidation in chemical and biological systems [2,3]. However DPDT did not offer any protection against TBARS production in phospholipids extract as shown in Table 3. The possible explanation for this difference in reactivity could be explained by the fact that DPDT may become redox active in brain homogenate but not in phospholipids extract. This may also indicate that brain homogenates is important to metabolize diphenyl ditelluride to an anti-oxidant intermediate, or alternatively brain homogenate may be important to transfer telluroxide formed after tellurium oxidation back to telluride.

We expected that the reduced form of DPDT can be a good reducing agent, one that would be readily reactive towards ROS. In addition, reversibility of electron transfer also points towards the compound's ability to 'redox cycle', *i.e.*, to participate in redox catalysis. However, (Contrary to our expectations) we did not find any alteration in the anti-oxidant activity of DPDT in brain homogenate at all studied pH values as evident from IC_{50} values shown in Table 4. Although the phenomenon of redox behavior, changes in anti-oxidant potential in different physiological conditions and in different cell cultures or oxidative stress models is therefore only just emerging, the results obtained so far from these experiments support the notion that DPDT is a redox active compound and can protect against TBARS production at all studied pH values.

Table 4
IC_{50} (μmol/l) values for basal and Fe(II) induced TBARS production for DPDT.

pH	Basal (μM)	Fe(II)-induced (μM)
5.4	1.32 ± 0.01	1.36 ± 0.03
5.8	1.30 ± 0.03	1.33 ± 0.01
6.4	1.31 ± 0.03	1.39 ± 0.02
6.8	1.29 ± 0.02	1.39 ± 0.01
7.4	1.21 ± 0.01	1.31 ± 0.01

In fact, literature data have demonstrated that tellurides can have pharmacological activity. In a variety of tumor models in mice and humans Ammonium Trichloro(dioxoethylene-*o*,*o*′)tellurate (AS101) had a clear anti-tumoral effect [40]. Phase I clinical trials with advanced cancer patients treated with AS101 showed increased production and secretion of a variety of cytokines, leading to a clear dominance in TH1 responses with a concurrent decrease in the TH2 responses [41]. While, Phase II clinical trials with cancer patients treated with AS101 in combination with chemotherapy have been initiated and completed, showing that treatment with AS101, with no major toxicity, induced a significant reduction in the severity of neutropenia and thrombocytopenia that accompany chemotherapy [42].

In the present work, we demonstrated for the first time that DPDT can protect Fe(II) induced TBARS production in brain homogenates at all studied pH values. In spite of this, the possible potential therapeutic use of DPDT should be questioned, since it is highly neurotoxic [11–14,43–45]. We have demonstrated that the pH of the tissue can modulate the ability of iron to participate in lipid peroxidation reactions. Apart from that we have shown that acidic pH also enhances detrimental effect of iron. Our observations will be of importance in our understanding of pathologies which are associated with low tissue pH. These studies confirm that DPDT and its reduced form is redox active within physiologically relevant potential range. The implication from these results for a biological system is that DPDT reacts with thiols on the basis of their chemical reactivity. If it is selective, accessibility or other molecular features may be more important determinants. Furthermore other aspect that deserve investigation is to determine a possible relationship between thiol-peroxidase activity of DPDT with the capacity of this compound in catalyzing thiol/sulfide exchange, and how these two chemical properties of tellurides correlates with their toxicological and pharmacological effects.

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4.3. 3rd Manuscript

Enhancement of iron-catalyzed lipid peroxidation by acidosisin brain homogenate: Comparative effect of diphenyl diselenide and ebselen

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Research Report

Enhancement of iron-catalyzed lipid peroxidation by acidosis in brain homogenate: Comparative effect of diphenyl diselenide and ebselen

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ABSTRACT

Iron is more soluble at lower pH values; therefore we hypothesized that decreasing the environmental pH would lead to increased iron-mediated lipid peroxidation. Diphenyl diselenide and ebselen are potential candidates as neuroprotective agent, particularly in situations involving overproduction of free radicals and involving cellular pH fall. The aim of the present study was (a) to investigate the relationship between lipid peroxidation and acidosis in brain homogenate and (b) to test the influence of pH on the antioxidant properties of diphenyl diselenide and ebselen. For the purpose rat brain homogenate was incubated at different pH ranging from physiological to acidic values and extent of lipid peroxidation was measured. Thiobarbituric acid-reactive species (TBARS) production significantly increased when homogenate was incubated in the pH (5.4–6.8) medium both in the absence and presence of Fe (II) as compared with physiological pH (7.4). These data indicate that lipid peroxidation processes, mediated by iron, are enhanced with decreasing extracellular pH. The iron mobilized may come from reserves where it is weakly bound. Diphenyl diselenide significantly protected TBARS production at all studied pH values while ebselen offered only a small statistically non-significant protection. However, calculated IC_{50} for TBARS inhibition indicated that pH did not change anti-oxidant activities of the tested compounds. This study provides in-vitro evidence for acidosis induced oxidative stress in brain homogenate and anti-oxidant action of diphenyl diselenide.

1. Introduction

There are a few pathologies that are associated with oxidative stress as well as low tissue pH values (Wike-Hooley et al., 1984; Arndt et al., 1998). Similarly, there is also considerable amount of evidence showing that during ischemia (brain, liver and kidney) intracellular pH can fall to values close to 6.0 (Uhlmann et al., 2004; Brooks et al., 2007; Harald et al., 2000; Obrenovitch et al., 1990). This decrease in pH can result in an enhancement of free radical formation, which is likely to result from lowered pH-induced iron delocalization from inactive to active redox sites (Rehncrona et al., 1989). Free

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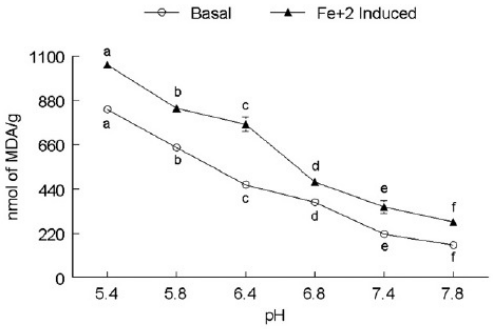

Fig. 1 – Effect of pH on basal and Fe (II)-induced (20 μM) TBARS production in supernatants of homogenates from brain of rats. Low-speed supernatant (S1) from tissue was incubated for 60 min in sodium phosphate buffer of different pH. TBARS are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n = 5–7). Different letters show main effect of pH at $p < 0.05$.

iron can easily stimulate fenton reaction which in turn can stimulate lipid peroxidation and oxidation of proteins and DNA (Valko et al., 2006). Thus, the fall in intracellular pH that is associated with ischemia cannot only influence metabolic processes, but it can potentiate or act sinergically with oxidative stress contributing to increased cellular injury.

The developments of new drugs that preserve neuronal integrity, acting after ischemic insult are under current investigation and a variety of seleno organic compounds are now considered potential antioxidant and chemopreventive agents (Commandeur et al., 2001). In line with this, ebselen (pz 51; 2-phenyl-1,2-benzisoselenazol-3(2H)-one), a synthetic selenium-containing heterocycle has been used experimentally and clinically with success in a variety of situations where free radicals are involved (Klotz and Sies, 2003, Nogueira et al., 2004). Diselenides are good candidates to become therapeutic antioxidant agents because they have some chemical and biochemical characteristics in common with ebselen, i.e., they exert glutathione peroxidase-like activity and can react with –SH groups, forming selenosulfide and selenol (–SeH), which is a pontent nucleophyle moiety (Engman et al., 1989; Farina et al., 2002). Diphenyl Diselenide (DPDS or $(PhSe)_2$), the simplest diaryl diselenide, is particularly important as a potential antioxidant drug in view to the fact that it has been shown to be more active as a glutathione peroxidase mimic (Wilson et al., 1989) and less toxic to rodents than ebselen (Klotz and Sies, 2003), and has also low toxicity for non-rodent mammals after long term exposure (Meotti et al., 2004).

Although several mechanisms have been proposed to explain the observed GPx activity of ebselen and DPDS, the available information reveals a hypothetical catalytic cycle in which, the Se-N bond of ebselen is readily cleaved by thiols to produce the corresponding selenenyl sulfides, which upon reduction by excess thiols produces selenol. Finally, the selenol reacts with organic hydroperoxide to form ebselen via ebselen–selenic acid (Maiorino et al., 1988). Similarly, (for DPDS) in the presence of excess thiol, the diselenide (RSeSeR) is reduced to the selenolate RSe^{-1} with the formation of RSSeR (1). Nucleophilic attack of thiol on RSSeR produces the disulfide and the 2nd selenolate RSe^{-1} ion (2) which further in the presence of oxygen produces diselenide (3).

$$R'SH + RSeSeR \leftrightarrow R'SSeR + RSe^{-1} + H^{+} \quad (1)$$

$$R'SSeR + R'SH \leftrightarrow R'SSR + RSe^{-1} + H^{+} \quad (2)$$

$$2RSeH \rightarrow RSeSeR \quad (3)$$

Since, selenols exist mostly in anionic form at neutral pH and represent good reducing groups under normal physiological conditions (Burk, 1994). From a hypothetical point of view the formation of stables selenolate (Se^{-1}) ions can increase the reducing properties of these moieties on the organochalcogenides and hypothetically can increase their antioxidant properties. However, there is no data in the literature supporting this assumption. Thus, to get a deeper insight about the potential use of organochalcogens as pharmacological agents for the treatment of ischemia, we determined the influence of pH on the in vitro anti-oxidant activity of DPDS and ebselen in brain homogenates. We have investigated whether DPDS and ebselen could protect brain homogenates incubated with Fe (II) in vitro at different pH values.

2. Results

2.1. *Effect of pH on basal and or Fe (II)-induced TBARS production in brain homogenate*

Two-way ANOVA (i.e. 6 (pHs))×2 (basal/iron) of TBARS levels in brain homogenates revealed significant main effect of pH and Fe (II) and also a significant Fe × pH interaction ($p < 0.05$). Indeed, basal and Fe (II) induced TBARS production significantly increased by decreasing pH and this effect was more pronounced at pH 5.4 and 5.8 ($p < 0.05$) as shown in Fig. 1.

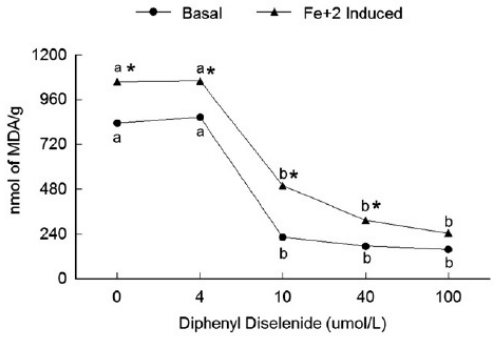

Fig. 2 – Effect of DPDS on basal or Fe (II)-induced (20 μM) TBARS production in supernatant of homogenate from brain of rats at pH 5.4. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n = 5–7). Different letters show main effect of DPDS, while asterisk/s shows main effect of Fe (II) at $p < 0.05$.

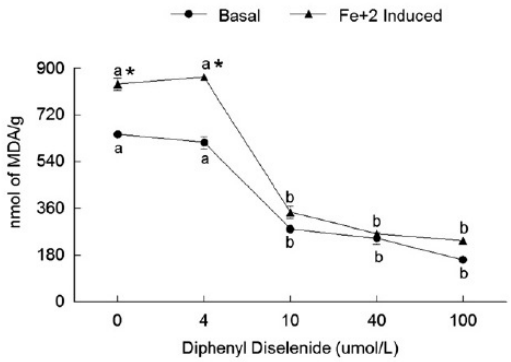

Fig. 3 – Effect of DPDS on basal or Fe (II)-induced (20 μM) TBARS production in supernatant of homogenate from brain of rats at pH 5.8. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n = 5–7). Different letters show main effect of DPDS, while asterisk/s shows main effect of Fe (II) at $p < 0.05$.

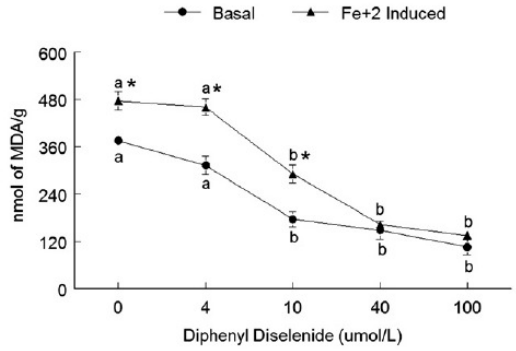

Fig. 5 – Effect of DPDS on basal or Fe (II)-induced (20 μM) TBARS production in supernatant of homogenate from brain of rats at pH 6.8. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n = 5–7). Different letters show main effect of DPDS, while asterisk/s shows main effect of Fe (II) at $p < 0.05$.

2.2. *Effect of DPDS on TBARS production in brain homogenates*

Three way ANOVA of TBARS production i.e. 6 pHs × 5 concentrations of DPDS × 2 (basal/iron) revealed a significant main effect of pH, DPDS and Fe (II) and also significant pH × Fe (II) × DPDS interaction ($p < 0.05$).

For the sake of clarity we have performed two-way analysis at each specific pH value.

Two way ANOVA (i.e. pH × basal/iron) for TBARS production revealed a significant main effect of Fe (II), DPDS and a significant Fe(II) × DPDS interaction at all studied pH values ($p < 0.05$). It is apparent from the (Figs. 2–7) that DPDS significantly protected ($p < 0.05$) both basal and iron-induced lipid peroxidation at all stated pH values.

2.3. *Effect of ebselen on TBARS production in brain homogenates*

Three-way ANOVA (i.e. 6 (pH)) × 5 concentrations of ebselen × 2 (basal/iron)) of TBARS production revealed a significant third-order interaction ($p < 0.05$). Interaction was significant because, as can be seen in Table 1 that low pH (i.e. 5.4–6.8, respectively), alone increased basal TBARS production as compared to physiological pH (7.4). In the same way, Fe (II) caused a marked increase in TBARS production at all studied pH values (Table 1).

For the sake of clarity we have performed two-way analysis at each specific pH value.

Two way ANOVA (i.e. pH × basal/iron) for TBARS production in case of ebselen revealed a significant main effect of Fe (II)

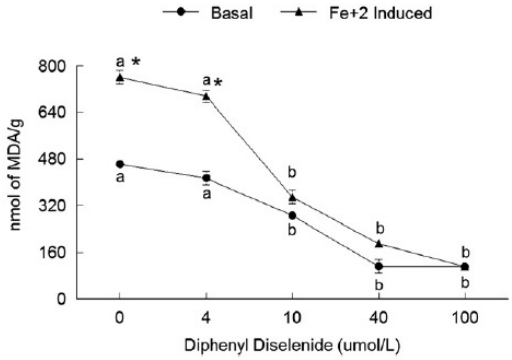

Fig. 4 – Effect of DPDS on basal or Fe (II)-induced (20 μM) TBARS production in supernatant of homogenate from brain of rats at pH 6.4. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n = 5–7). Different letters show main effect of DPDS, while asterisk/s shows main effect of Fe (II) at $p < 0.05$.

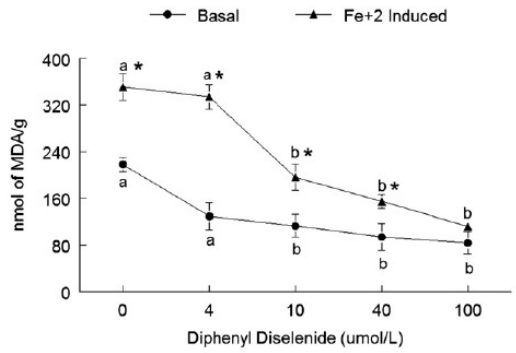

Fig. 6 – Effect of DPDS on basal or Fe (II)-induced (20 μM) TBARS production in supernatant of homogenate from brain of rats at pH 7.4. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n = 5–7). Different letters show main effect of DPDS, while asterisk/s shows main effect of Fe (II) at $p < 0.05$.

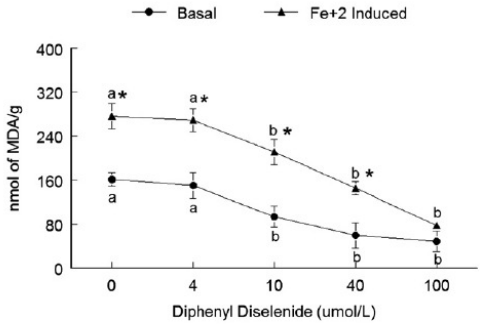

Fig. 7 – Effect of DPDS on basal or Fe (II)-induced (20 μM) TBARS production in supernatant of homogenate from brain of rats at pH 7.8. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n=5–7). Different letters show main effect of DPDS, while asterisk/s shows main effect of Fe (II) at $p<0.05$.

and at all studied pH values ($p<0.05$). It is apparent from the (Table 1) that ebselen exerted a small non-significant ($p<0.05$) antioxidant effect on basal and iron-induced lipid peroxidation at all stated pH values.

3. Discussion

The results presented here show that lipid peroxidation in brain homogenate in the absence of Fe (II) is enhanced upon a shift in the pH of the incubation solutions from physiological conditions (pH 7.4) to acidic ones (pH 5.4) as shown in Fig. 1. Similarly, the amount of TBARS produced by incubation of homogenate with Fe (II) alone at pH 7.4 was lower. However, as the pH of the solution pH was decreased from 7.4 to 5.4, Fe (II) dependent TBARS production markedly increased (Fig. 1).

The enhancement of pH dependent lipid peroxidation can be attributed to mobilized iron which may come from reserves where it is weakly bound. The mechanisms of iron release during ischemia are likely complex, potentially involving both intracellular stores and intravascular iron transport and delivery to brain. Because of the potential cytotoxicity of iron, physiological systems have developed intricate mechanisms to protect cells from oxidative injury. Brain iron is largely complexed to protein carriers (transferrin, lactoferrin) for intravascular and transmembrane transport and to ferritin for intracellular storage. Ischemic acidosis may drive iron dissociation from those carrier proteins with pH-dependent binding characteristics (Bates and Schlaback, 1975).

It has been shown that the protein transferrin carries two iron ions, although it is normally only about one third saturated with iron (Welch, 1990). Transferrin loses its bound iron at acidic pH. The initial 10% of iron in saturated human transferrin is lost at a pH of 5.4 and the final 10% at a pH of 4.3 (Welch, 1990). Whereas if transferrin is bound to its receptor, essentially all the iron is released at pH 5.6–6.0 (Sipe and Murphy, 1991). The pH-dependent affinity of transferrin for iron decreases under acidic conditions leading to dissociation of iron from transferrin and other proteins like ferritin and lactoferrin (Balla et al., 1990). Once mobilized, free iron likely binds nonspecifically to a variety of small molecular moieties and augments the ordinarily small low molecular weight (LMW) nonprotein-bound tissue pool. In cortical homogenates, striking increases in LMW iron are observed at pH 6.0 when pH is reduced from 7.0 by direct addition of lactic acid. Furthermore, brain from decapitated hyperglycemic rats shows elevated LMW iron relative to normoglycemic controls (Hurn et al., 1991).

The mobilized iron Fe (II) can interact with enzymatically and/or non-enzymatically generated superoxide ($O_2^{\cdot -1}$) (Haber–Weiss reaction) and/or hydrogen peroxide (H_2O_2) (Fenton reaction) (Liochev and Fridovich, 2002; Koppenol, 2001) producing reactive oxygen species. In fact, $O_2^{\cdot -1}$ and H_2O_2 may be produced directly from dissolved oxygen (O_2) in

Table 1 – Effect of ebselen on basal or Fe (II)-induced (20 μM) TBARS production in supernatants of homogenates from brain of rats

Treatment	pH					
	5.4	5.8	6.4	6.8	7.4	7.8
Basal(B)	834±13	645±12	462±24	375±9	218±16	161±12
B+4 μM	801±24	612±31	443±34	364±16	219±25	179±23
B+10 μM	798±31	575±31	424±39	347±12	187±21	151±31
B+40 μM	778±23	584±25	402±27	351±21	194±19	137±14
B+100 μM	764±17	579±19	387±21	331±9	169±15	129±10
Fe(II)-Induced *	1056±38	839±39	761±37	476±24	351±31	276±16
Fe(II)+4 μM*	1041±21	822±31	749±41	467±31	367±39	274±23
Fe(II)+10 μM*	1012±37	819±45	730±31	441±27	349±24	258±21
Fe(II)+40 μM*	985±23	801±21	712±28	427±41	331±19	240±29
Fe(II)+100 μM*	951±41	778±16	698±23	421±22	319±27	223±15

Low-speed supernatant (S1) from tissue was incubated for 60 min in a medium containing sodium phosphate buffer at different pH (i.e. 5.4, 5.8, 6.4, 6.8, 7.4 and 7.8). The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n=5–7). Asterisk/s shows main effect of Fe (II) at $p<0.05$.

aqueous media in the Fe^{2+}-mediated basal/autoxidation reactions as follows:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot -1} \quad (4)$$

The dismutation of superoxide to hydrogen peroxide and oxygen has been shown to be faster at acidic pH (Halliwell and Gutteridge, 1989).

$$HO_2^{\cdot} + O_2^{\cdot -1} + H^+ \rightarrow H_2O_2 + O_2 \quad (5)$$

The H_2O_2 and superoxide produced in above reactions (4 and 5) may react together in a metal catalyzed (Haber–Weiss reaction) to produce the extremely reactive hydroxyl radical, which may then abstract hydrogen atoms from polyunsaturated fatty acids.

$$Fe^{2+} + O_2^{\cdot -1} + 2H^+ \rightarrow Fe^{3+} + H_2O_2 \quad (6)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- 1 + OH^{\cdot} \quad (7)$$

Ferrous ion (Fe (II)) is the form of iron that is capable of redox cycling. Oxidation of Fe (II) to Fe (III) resulting in ROS formation is greatly dependent upon the pH of the media. The possibility of reduction of Fe (III) to Fe (II) by interaction with $O_2^{\cdot -1}$ at the early phase of lipid peroxidation under acidic conditions, perhaps via an intermediate, perferryl iron (Pederson et al., 1973) cannot be excluded.

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot -1} \quad (8)$$

$$Fe^{3+} + O_2^{\cdot -1} \rightarrow [Fe^{3+}\text{–}O_2^{\cdot -1} \leftrightarrow Fe^{2+}\text{–}O_2] \leftrightarrow Fe^{2+} + O_2 \quad (9)$$

This pH environment and a series of chain reactions seem to provide optimal conditions for maximal catalytic efficiency of iron. Thus, the acidic pH not only release iron from "safe" site but also potentiate the pro-oxidant effect of Fe (II) as we observed from significant increase in TBARS production at pH (6.8–5.4) (Fig. 1).

The antioxidant properties of DPDS have been tested in many studies where the compound demonstrated a protective potential against oxidative insults in tissues such as lung (Luchese et al., 2007), blood (Posser et al., 2006), brain (Rossato et al., 2002) and liver (Borges et al., 2006). However, there are no studies on the antioxidant activity of this compound at different pH values. DPDS significantly reduced both basal and Fe (II) induced TBARS at all studied pH values in brain homogenate (Figs. 2–7). Several investigators have proposed some iron–oxygen complexes such as the ferryl ion (Bors et al., 1979), perferryl ion (Pederson et al., 1973), and Fe^{+2}–O_2–Fe^{+3} complex (Bucher et al., 1983) for oxidizing species of Fe (II) other than $O_2^{\cdot -1}$, H_2O_2 and $^{\cdot}OH$ which are involved in the initiation of Fe(II)-dependent lipid peroxidation. A plausible mechanism by which diphenyl diselenide is conferring protective action against Fe (II)-induced lipid peroxidation in these homogenate is that diphenyl diselenide could not only be operating as a scavenger, but may also be interacting directly with Fe (II) or its oxidized forms. Ebselen, on the other hand did not offer any protection against TBARS production at none of the studied pH as shown in Table 1. Importantly, data from several studies shows that thiol peroxidase activity of synthetic organoselenium compounds are much higher than that presented by ebselen [11]. When the diselenide bond of diphenyl diselenide is disrupted, two selenols can be yielded, differently from ebselen, improving the catalytic reaction that is of particular significance to living cells. Despite the fact that DPDS has very low oxidation (Epa/mV −741) and reducing potential (E_{pc}/mV −799) (Collins et al., 2005) and that the reduced form of the compound can therefore be expected to be a good reducing agent, one that would be readily reactive towards ROS. In addition, reversibility of electron transfer also points towards the compound's ability to 'redox cycle', i.e., to participate in redox catalysis. However, (contrary to our expectations) we did not found any alteration in the anti-oxidant activity of DPDS at all studied pH values as evident from the IC_{50} values listed in Table 2. Although the phenomenon of redox behavior, changes in anti-oxidant potential in different physiological conditions and in different cell cultures or oxidative stress models is therefore only just emerging, the results obtained so far from these experiments support the notion that DPDS is a redox active compound can protect against TBARS production at all studied pH values.

In the present work, we demonstrated for the first time the antioxidant effect of DPDS on Fe (II) induced lipid peroxidation in brain homogenate in vitro not only at physiological pH but also under acidic conditions. We have demonstrated that the pH of the tissue can modulate the ability of iron to participate in lipid peroxidation reactions. Ferrous iron amplifies oxidative damage to lipids at low pH. Apart from that we have shown that acidic pH also enhances detrimental effect of iron. Our observations will be of importance in our understanding of pathologies which are associated with low tissue pH. These studies confirm that DPDS and its reduced form is redox active within physiologically relevant potential range. Although the observation in the present study cannot be directly related to in vivo conditions, it seems that the results may give us a clue to understand the role of Fe (II) in the iron-mediated cell injury and/or diseases under acidic conditions and a possible anti-oxidant effect of DPDS in ischemic/reperfusion injury. Furthermore other aspect that deserve investigation is to determine a possible relationship between thiol-peroxidase activity of DPDS with the capacity of this compound in catalyzing thiol/diselenide exchange, and how these two chemical properties of selenides correlates with their toxicological and pharmacological effects.

4. Experimental procedures

4.1. *DPDS and ebselen, synthesis and preparation*

DPDS and ebselen [2-phenyl-1,2-benzisoselenazol-3(2H)-one] were synthesized according to literature methods (Paulmier, 1986, Engman et al., 1989). Analysis of the ^{1}HNMR and ^{13}CNMR spectra showed that both compounds obtained presented analytical and spectroscopic data in full agreement with their assigned

Table 2 – IC_{50} (μmol/l) values for basal and Fe(II) induced TBARS production for DPDS

pH	Brain	
	Basal (μM)	Fe (II)-Induced (μM)
5.4	8.3±0.9	9.4±1.12
5.8	8.1±1.1	7.4±2.24
6.4	11±0.8	10.1±0.87
6.8	9.7±0.8	11.3±0.68
7.4	9.4±0.6	10.9±0.99
7.8	9.4±0.87	13.8±1.2

structures. The chemical purity of the compounds (99.9%) was determined by GC/HPLC.

4.2. *Preparation of buffers*

All buffers were prepared at room temperature with constant ionic strength. Buffer solutions were maintained at 4 °C until the initiation of the experiments. Direct measurement of pH values in the tubes at 37 °C verified that actual pH values were typically within ±0.05. The final buffer concentration in all experiments was 10 mM.

4.3. *Animals*

(12–14) Adult male Wistar rats from our own breeding colony (250–350 g) were maintained in an air-conditioned room (22–25 °C) under natural lighting conditions, with water and food (Guabi, RS, Brazil) ad libitum. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil.

4.4. *Tissue preparation*

Animals were anesthetized with ether and killed by decapitation. Brain was quickly removed, placed on ice, and homogenized within 10 min, in 10 mmol/l Tris/HCl buffer, pH 7.4 (in 10 volume). The homogenate was centrifuged at 4000 ×*g* at 4 °C for 10 min to yield a low speed supernatant fraction (S1) that was used immediately for TBARS assay (Puntel et al., 2007).

4.5. *Lipid peroxidation assay*

Lipid peroxidation was determined by measuring thiobarbituric acid-reactive species (TBARS) as described by Ohkawa et al. (1979) with minor modifications (Puntel et al., 2007). Tissue homogenate was prepared by homogenization as described above. Aliquots of the brain homogenate (100 μl) was incubated for 60 min in a medium containing 10 mM sodium phosphate buffer of different pH ranging from 5.4 to 7.8 (pH was checked after the addition of brain supernatants both at the beginning and at the end of the incubation period) and in the presence of other reagents at concentrations indicated in the legends. The mixtures were incubated at 37 °C for 60 min. The reaction was stopped by addition of 0.5 ml of acetic acid buffer and lipid peroxidation products were measured by the addition of 0.5 ml of TBA 0.6% and 0.2 ml of SDS 8.1%. The color reaction was developed by incubating tubes in boiling water for 60 min. TBARS levels were measured at 532 nm using a standard curve of MDA. The values are expressed in nmol MDA/g of tissue of 5–7 experiments. Where indicated, solutions of $FeSO_4$ were made just before use in distilled water.

4.6. *IC_{50} calculation*

IC_{50} (concentration inhibiting 50% of lipid peroxidation) for lipid peroxidation was determined by the method of Dixon and Webb (1964).

4.7. *Statistical analysis*

The results are expressed as the mean ± standard error (SEM). Data were analyzed statistically by analysis of variance (i.e. two and three way ANOVA), followed by univariate analysis and Duncan's multiple range test when appropriate.

Acknowledgments

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4.3. 4th Manuscript

Effects of acidosis and Fe (II) on lipid peroxidation in phospholipid extract: Comparative effect of diphenyl diselenide and ebselen

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Short communication

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ABSTRACT

The influence of acidosis on lipid peroxidation in phospholipids extract from egg yolk was studied. In addition, we have also tested the effect of pH on the anti-oxidant properties of diphenyl diselenide and ebselen. Acidosis increased rate of lipid peroxidation both in the absence and presence of Fe (II). Diphenyl diselenide significantly protected TBARS production at all studied pH values while ebselen offered only a small statistically non-significant protection. However, changing the pH of the reaction medium did not alter the anti-oxidant activity of the tested compounds. This study provides in vitro evidence for acidosis-induced oxidative stress in phospholipids extract and anti-oxidant action of diphenyl diselenide.

1. Introduction

Lipid peroxidation in cell membrane phospholipids induced by reactive oxygen species (ROS) and/or free radicals leads to membrane damage and has been proposed to be a major mechanism for the onset of several pathological events *in vivo* including postischemic-reperfusion injury, cancer, Parkinson's disease, senile dementia and aging (Halliwell and Gutteridge, 1984). Ebselen (pz 51; 2-phenyl-1,2-benzisoselenazol-3(2H)-one), a synthetic selenium-containing heterocycle has been used experimentally and clinically with success in a variety of situations where free radicals are involved (Nogueira et al., 2004). Diphenyl diselenide (DPDS or $(PhSe)_2$), the simplest diaryl diselenide, is particularly important as a potential anti-oxidant drug in view to the fact that it has been shown to be more active as a glutathione peroxidase mimic (Wilson et al., 1989) and has also low toxicity for non-rodent mammals after long term exposure (Meotti et al., 2004). To explain the observed GPx activity of ebselen and DPDS, the available information reveals a hypothetical catalytic cycle in which, the Se–N bond is readily cleaved by thiols to produce the corresponding selenenyl sulfides, which upon reduction by excess thiols produce selenol. From a hypothetical point of view the formation of stables selenolate (Se^{1-}) ions can increase the reducing properties of these moieties on the organochalcogenides and hypothetically can increase their anti-oxidant properties. However, there is no data in the literature supporting this assumption. Thus, to get a deeper insight about the potential use of organochalcogens as pharmacological agents, we determined the influence of pH on either basal or iron-induced TBARS production in vitro at different pH ranging from low (acidic) to physiological value in phospholipids extract from egg yolk.

2. Materials and methods

2.1. Chemicals

DPDS and ebselen [2-phenyl-1,2-benzisoselenazol-3(2H)-one] were synthesized according to literature methods (Paulmier, 1986; Engman et al., 1989). Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that both compounds obtained presented analytical and spectroscopic data in full agreement with their assigned structures. The chemical purity of the compounds (99.9%) was determined by GC/HPLC. All other chemicals were purchased from standard suppliers.

2.2. Phospholipids extraction

The phospholipids were extracted from eggs by a solution 3:2 of hexane–isopropanol in the proposition of 1 g of egg to 10 ml of this solution. The mixture was filtered and put in the rata-vapor with the maximum temperature of 60 °C. Extract was weighed and dissolved in distilled water in the proposition of 10 mg of extract to 10 ml of water which was used for TBARS assay.

2.3. Lipid peroxidation assay

Lipid peroxidation was determined by measuring thiobarbituric acid-reactive species (TBARS) as described by Ohkawa et al. (1979). Aliquots of 100 μl from extract was incubated for 60 min in a medium containing 10 mM sodium phosphate buffer, of different pH ranging from 5.4 to 7.4, i.e. 5.4, 5.8, 6.4, 6.8, 7.4 and 7.8 and in the presence of other reagents at concentrations indicated in the legends. The mixture was incubated at 37 °C in a water bath for 60 min. The reaction was stopped by the addition of 0.5 ml of acetic acid buffer and lipid peroxidation products were measured by the addition of 0.5 ml of TBA 0.6% and 0.2 ml of SDS 8.1%. The color

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reaction was developed by incubating tubes in boiling water for 60 min. 2 ml of n-butanol was added to initial mixture and was centrifuged for 10 min at 4000 × *g* at 4 °C. TBARS levels were measured at 532 nm using a standard curve of MDA. The values are expressed in nmol MDA/g of extract. Where indicated, solutions of $FeSO_4$ were made just before use in distilled water.

2.4. Statistical analysis

The results are expressed as the mean ± standard error (S.E.M.). Data were analyzed statistically by analysis of variance, i.e. two- and three-way ANOVA, followed by univariate analysis and Duncan's multiple range test when appropriate. Differences between groups were considered significant when $p < 0.05$.

3. Results

3.1. Effect of pH on basal and/or Fe (II)-induced TBARS production in phospholipid extract

Two-way ANOVA of Fe (II)-induced TBARS levels in phospholipids extract revealed significant main effect of pH and Fe (II) and also a significant Fe × pH interaction ($p < 0.05$). Indeed, basal and Fe (II)-induced TBARS production significantly increased by decreasing pH and this effect was more pronounced at pH 5.4 and 5.8 ($p < 0.05$) as shown in Fig. 1.

3.2. Effects of DPDS and ebselen on TBARS production in phospholipids extract

Three-way ANOVA of TBARS production, i.e. 6 pHs × 5 concentrations of DPDS × 2 (basal/iron) revealed a significant main effect of pH, DPDS and Fe (II) and also significant pH × Fe (II) × DPDS interaction ($p < 0.05$).

For the sake of clarity, data from the anti-oxidant effect of DPDS at different pH values and in the presence or absence of added Fe (II), which was analyzed by a three-way ANOVA, was further analyzed by two-way ANOVA at each pH.

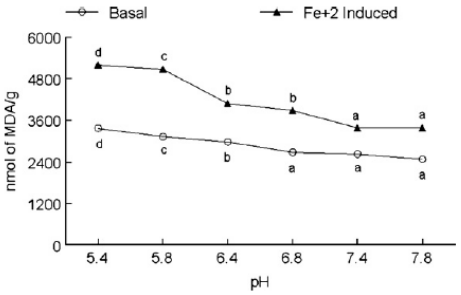

Fig. 1. Effect of pH on basal and Fe (II)-induced (20 μM) TBARS production in phospholipids extract from egg yolk at different pH. Data are expressed as means ± S.E.M. ($n = 5–7$). Different letters shows main effect of pH at ($p < 0.05$).

Two-way ANOVA for TBARS production at all studied pH revealed a significant main effect of Fe (II) and DPDS ($p < 0.05$) and also a significant Fe (II) × DPDS interaction. Interaction was significant because the pro-oxidant effect of Fe (II) was evident in the absence and presence of DPDS as shown in Table 1. It is possible to observe that Fe (II) increased TBARS production while DPDS reduced it.

Similarly, two-way ANOVA for TBARS production in case of ebselen revealed a significant main effect of Fe (II) at all studied pH values. It is apparent from Table 2 that ebselen exerted a small non-significant ($p < 0.05$) anti-oxidant effect on basal and Fe (II)-induced lipid peroxidation at all stated pH values.

Table 1
Effect of DPDS on basal or Fe (II)-induced (20 μM) TBARS production in supernatants of phospholipids extract from egg yolk at different pH. Data are expressed as means ± S.E.M. ($n = 5–7$).

Treatment	pH					
	5.4	5.8	6.4	6.8	7.4	7.8
Basal (B)[a]	3309 ± 112	3235 ± 78	2970 ± 121	2529 ± 139	2465 ± 79	2370 ± 111
B + 4 μM[a]	3058 ± 132	3179 ± 94	2716 ± 111	2559 ± 121	2416 ± 87	2320 ± 161
B + 10 μM[a]	3072 ± 87	3043 ± 112	2614 ± 121	2452 ± 130	2414 ± 121	2313 ± 151
B + 40 μM[b]	2710 ± 142	2752 ± 109	2126 ± 98	2361 ± 83	2126 ± 112	2023 ± 112
B + 100 μM[b]	1999 ± 154	2016 ± 142	2042 ± 87	1856 ± 79	1756 ± 90	1888 ± 87
Fe (II)-induced[a*]	5110 ± 114	5001 ± 181	4342 ± 91	3841 ± 151	3348 ± 131	3442 ± 141
Fe (II) + 4 μM[a*]	5082 ± 89	4988 ± 132	4006 ± 110	3351 ± 101	3197 ± 172	3288 ± 119
Fe (II) + 10 μM[a*]	4810 ± 121	4924 ± 87	3915 ± 121	3686 ± 98	3287 ± 152	3178 ± 129
Fe (II) + 40 μM[b*]	4398 ± 112	4484 ± 78	3295 ± 78	3211 ± 60	3304 ± 121	2976 ± 112
Fe (II) + 100 μM[b*]	3141 ± 132	3277 ± 101	3088 ± 101	2915 ± 54	2576 ± 101	2779 ± 98

Different letters shows main effect of DPDS.
[*] Main effect of Fe (II) at ($p < 0.05$).

Table 2
Effect of ebselen on basal or Fe (II)-induced (20 μM) TBARS production in supernatants of phospholipids extract from egg yolk at different pH. Data are expressed as means ± S.E.M. ($n = 5–7$).

Treatment	pH					
	5.4	5.8	6.4	6.8	7.4	7.8
Basal (B)	3404 ± 112	3001 ± 144	2974 ± 151	2819 ± 112	2782 ± 98	2589 ± 121
B + 4 μM	3054 ± 141	3042 ± 132	2704 ± 139	2819 ± 131	2685 ± 111	2567 ± 115
B + 10 μM	3148 ± 111	3068 ± 139	2743 ± 145	2715 ± 98	2688 ± 95	2447 ± 152
B + 40 μM	3299 ± 99	3020 ± 141	2668 ± 112	2672 ± 176	2599 ± 145	2485 ± 136
B + 100 μM	3154 ± 121	3001 ± 123	2601 ± 121	2657 ± 145	2501 ± 121	2339 ± 139
Fe (II)-induced[*]	5269 ± 145	5128 ± 176	3813 ± 149	3919 ± 131	3415 ± 187	3315 ± 110
Fe (II) + 4 μM[*]	5133 ± 129	5176 ± 134	3871 ± 137	3834 ± 154	3315 ± 131	3256 ± 121
Fe (II) + 10 μM[*]	5261 ± 101	5099 ± 151	3704 ± 101	3782 ± 139	3289 ± 123	3190 ± 143
Fe (II) + 40 μM[*]	5124 ± 98	4999 ± 111	3612 ± 142	3823 ± 126	3290 ± 143	3178 ± 141
Fe (II) + 100 μM[*]	5106 ± 112	4912 ± 142	3636 ± 137	3750 ± 131	3258 ± 111	3168 ± 111

[*] Main effect of Fe (II) at ($p < 0.05$).

4. Discussion

The results presented here show that lipid peroxidation in phospholipids extract in the absence of Fe (II) is enhanced upon a shift in the pH of the incubation solutions from physiological conditions (pH 7.4) to acidic ones (pH 5.4) as shown in Fig. 1. Similarly, the amount of TBARS produced by incubation of phospholipids extract with Fe (II) alone at pH 7.4 was lower. However, as the pH of the solution pH was decreased from 7.4 to 5.4, Fe (II)-dependent TBARS production markedly increased (Fig. 1). These results strongly suggest that the ability of Fe (II) to initiate lipid peroxidation is strengthened by increasing [H^+]. These data are consistent with the observation that lipid peroxidation rate of brain homogenate is stimulated by a decrease of pH from 7.4 to 6.5 (Funahashi et al., 1994). At present, two possibilities have been proposed to explain the onset of acidosis-associated ischemic disorders: (a) stimulation of intracellular oxygen free radical production (Rehncrona et al., 1989), resulting in an enhanced peroxidation of cellular components; and (b) facilitation of iron release from its protein binding sites (Bralet et al., 1992).

DPDS significantly reduced both basal and Fe (II)-induced TBARS at all studied pH values in a concentration-dependent manner in phospholipids extract as shown in Table 1. Data from the literature revealed the fact that Fe (II)-dependent TBARS production in phospholipids liposomes under acidic condition is not inhibited by the addition of SOD, catalase and •OH scavengers (mannitol, sodium benzoate and dimethylthiourea) (Ohyashiki and Nunomura, 2000). The lack of action of SOD, catalase and •OH scavengers in Fe (II)-dependent TBARS production suggests that other species rather than $O_2^{\bullet -}$, H_2O_2 and •OH are involved in the initiation of Fe (II)-dependent lipid peroxidation. Several investigators have proposed some iron–oxygen complexes such as the ferryl ion (Bors et al., 1979), perferryl ion (Pederson et al., 1973), and Fe^{2+}–$O_2^{\bullet}$–Fe^{3+} complex (Bucher et al., 1983), for oxidizing species of Fe (II). A plausible mechanism by which DPDS is conferring protective action against Fe (II)-induced lipid peroxidation in these extracts is that DPDS could not only be operating as a scavenger, but may also be interacting directly with Fe (II) or its oxidized forms. While it is apparent from Table 2 that the anti-oxidant effect of ebselen was very little and not significant ($p < 0.05$) at the highest concentration tested. Importantly, data from several studies shows that thiol peroxidase activity of synthetic organoselenium compounds are much higher than that presented by ebselen (Nogueira et al., 2004). When the diselenide bond of DPDS is disrupted, two selenols can be yielded, differently from ebselen, improving the catalytic reaction that is of particular significance to living cells. Despite the fact that DPDS has very low oxidation (E_{pa}/mV −741) and reducing potential (E_{pc}/mV −799) and that the reduced form of the compound can therefore be expected to be a good reducing agent, one that would be readily reactive towards ROS. However (contrary to our expectations), we did not find any alteration in the anti-oxidant activity (with respect to pH) of DPDS and ebselen at all studied pH values.

In the present work, we demonstrated for the first time the anti-oxidant effect of DPDS on Fe (II)-induced lipid peroxidation in phospholipids extract *in vitro* not only at physiological pH but also under acidic conditions. These results support the anti-oxidant potential of DPDS, as already demonstrated in several tissues, blood (Posser et al., 2006), brain (Rossato et al., 2002) and liver (Borges et al., 2006), which is extended to phospholipids extract in this study. These phenomena seem to be related to its thiol peroxidase like activity and also to a possible direct interaction with Fe (II) or its oxidized derivatives. Although the observation in the present study cannot be directly related to *in vivo* conditions, it seems that the results may give us a clue to understand the role of Fe (II) in the iron-mediated cell injury and/or diseases under acidic conditions and a possible anti-oxidant effect of DPDS in ischemic/reperfusion injury.

Conflict of interest

There is no conflict of interest in the conduct and reporting of research (e.g., financial interests in a test or procedure, funding by pharmaceutical companies for drug research).

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5.0 General comments / General discussion

5.1 Interaction profile of thiols with diphenyl diselenide

The behaviors of thiols towards diphenyl diselenide revealed the fact that cysteine has higher rate constant as compared to GSH and DTT at physiological pH (7.4). The difference in the rates of oxidation both in the absence and presence of $(PhSe)_2$ can be explained by the fact that both the extent of ionization of a thiol and the intrinsic nucleophilicity of the corresponding thiolate anion determine the overall reactivity of thiols in this type of reactions. It has been suggested that the lower the pKa of a thiol the lower the nucleophilicity of the thiolate but the higher the relative concentration of thiolate [48-49]. This means that at physiological pH the CYS with the lower pKa would be the better nucleophile than GSH and DTT in our experimental set-up. We have also observed that GSH has lower rate constant than DTT at pH 7.4, both in the absence and presence of $(PhSe)_2$. This difference can be explained by the fact that GSH is sterically more hindered than DTT. Secondly, the close spatial proximity of two thiol groups in DTT not only facilitates auto-oxidation, but also promotes rate of oxidation in the presence of $(PhSe)_2$. The lower rate constants of DMSA and DMPS can also be attributed to extreme steric hindrance by carboxyl groups and low nucleophilicity of these thiols.

The effect of pH on rate of reaction for CYS, DMPS and GSH revealed that these thiols are more reactive when ionized and which is observable by gradual increase of rate constant with increasing pH both in the absence and presence of $(PhSe)_2$. This indicates that the deprotonated form of these thiols is the active species. Our study further revealed that the apparent rate constants for the reaction of $(PhSe)_2$ with DMSA and DTT were higher at low pH. A possible explanation for the observed differences in pH dependence between DTT, DMSA and these thiols could not be that the thiolate anion form is the actual substrate for $(PhSe)_2$. This is underlined by the similar pH profiles of $(PhSe)_2$ reduction by other thiols. The question of higher reactivity at lower pH for carboxylic acid containing molecules may be related to a decrease in the reactivity of the negative charges as pH

lowers, i.e., the steric hindrance of the charges diminish as pH falls. While in case of DTT we propose that the reaction may be acid hydrolyzed.

5.2 Why low pH increased extent of lipid peroxidation?

Most studies (but not all) have shown that acidity increases the oxidation of lipids. The autoxidation of linoleic acid was increased by acidity (whereas that of methyl linoleate was decreased by acidity) [50] and the oxidation of polyunsaturated fatty acids by iron–ascorbate , by ferritin–ascorbate or by tissue homogenates [51] was much faster at acidic pH. The oxidation brain homogenates [52] or brain slices [53] were increased greatly at acidic pH. Phospholipid liposomes are oxidised faster at acidic pH by human activated neutrophils [54] or by autoxidation [55]. In contrast, the autoxidation of phospholipid emulsions has been reported to be slower at acidic pH [56].

Aliquots (100 µl) of tissue homogenates were incubated for 60 min in a medium contains 10 mM sodium phosphate buffer, of different pH ranging from 5.4 to 7.8. The rate of per oxidation exhibits a dramatic increase as the pH is decreased from 7.8 to 5.4 both in the presence and absence of Fe (II) in tissue homogenates. The enhancement of pH dependent lipid peroxidation can be attributed to mobilized iron which may come from reserves where it is weakly bound. The pH dependency of TBARS production can be explained by the fact, firstly, that the protein transferrin carries two iron ions, although it is normally only about one third saturated with iron [57] Transferrin loses its bound iron at acidic pH. The pH-dependent affinity of transferrin for iron decreases under acidic conditions leading to dissociation of iron from transferrin and other proteins like ferritin and lactoferrin [58].

The mobilized iron Fe (II) can interact with enzymatically and/or non-enzymatically generated superoxide ($O_2^{\bullet -1}$) (Haber–Weiss reaction) and/or hydrogen peroxide (H_2O_2) (Fenton reaction) [59] producing reactive oxygen species.

In fact, $O_2^{\bullet -1}$ may be produced directly from dissolved oxygen (O_2) in aqueous media in the Fe^{2+}-mediated basal/autoxidation reactions as follows:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -1} \qquad (1)$$

The superoxide produced in above reactions (1) may react with Fe (II) in a metal catalyzed (Haber–Weiss reaction) to produce the extremely reactive hydroxyl radical, which may then abstract hydrogen atoms from polyunsaturated fatty acids.

$Fe^{2+} + O_2^{\bullet -1} + 2H^+ \rightarrow Fe^{3+} + H_2O_2$ (2)

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-1} + OH^{\bullet}$ (3)

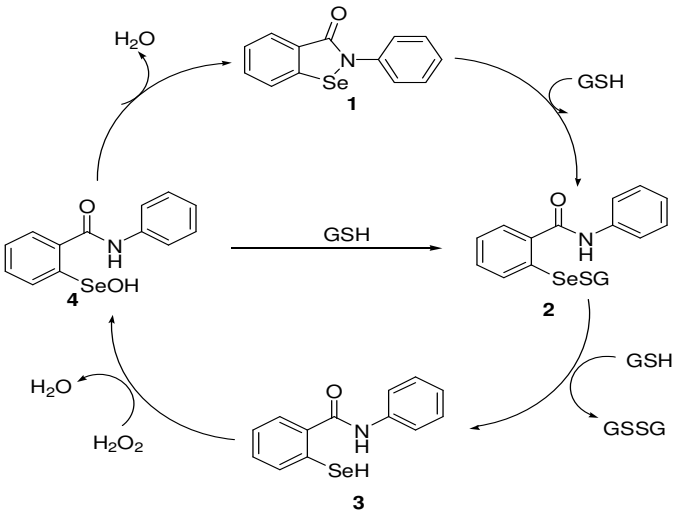
Indeed, transferrin has been shown to release bound iron during in vitro acidotic conditions, particularly as pH falls to <6.0 and brain cortical homogenates media release iron under acidic conditions [54]. Furthermore, acidosis increases TBARS in anoxic brain slices [53], brain homogenate and phospholipids liposome [60].

To support the mechanism and to explore the involvement of Fe (II) especially at low pH, we added iron chelator i.e. desferoxamine, DFO (1mM) in the absence of extracellular added iron at acidic values. The addition of DFO inhibited TBARS production at acidic pH values indicating the Fe (II) release and its participation in lipid peroxidation processes. The data presented here provides direct evidence for enhanced lipid peroxidation in rat's tissue homogenates at acidic pH which are consistent with our previous observations [61]. In the same way, acidic pH enhances the detrimental effect of iron. In our study we have addressed the catalytic efficiency of iron in tissue homogenates as the environmental pH changes. Thus, acidic pH not only releases iron from "safe" sites, but also potentiate the pro-oxidant effect of Fe (II) as apparent from increased lipid peroxidation.

5.3 Differences in activities of organochalcogens

DPDS significantly reduced both basal and Fe (II) induced TBARS at all studied pH values in tissue homogenates. Surprisingly, ebselen did not offer any protection against TBARS production at none of the studied pH neither in tissue preparation.

To explain the difference between the observed activities of the these two organoselenium compounds, we can take help from literature in which the available information reveals a hypothetical catalytic cycle in which, the Se-N bond of ebselen (1) is readily cleaved by thiols to produce the corresponding selenenyl sulfides (2), which upon reduction by excess thiols produces selenol (3). Finally, the selenol reacts with organic hydroperoxide (produced in reaction 2) to form ebselen via ebselen-selenic acid (4) as shown in (Scheme-1) [62].



Similarly, (for DPDS) in the presence of excess thiol, the diselenide (RSeSeR) is reduced to the selenolate RSe^{-1} with the formation of RSSeR (5). Nucleophilic attack of thiol on RSSeR produces the disulfide and the 2nd selenolate RSe^{-1} ion

$$R'SH + RSeSeR \leftrightarrow \quad R'SSeR + RSe^{-1} + H^{+} \qquad (4)$$

$$R'SSeR + R'SH \leftrightarrow R'SSR' + RSe^{-1} + H^{+} \qquad (5)$$

The two selenolate molecules produced in above reactions (4,5) can react with H_2O_2 (produced in reaction.2) production selenic acid (reaction-6) which upon reactions with GSH produces selenyl sulfide (reaction-7) and regenerate a molecule of diphenyl diselenide (reaction 8).

$$2\ RSe^{-1} + H_2O_2 \rightarrow 2\ RSeOH \qquad (6)$$

$$2\ RSeOH + 2GSH \rightarrow 2RseSG \qquad (7)$$

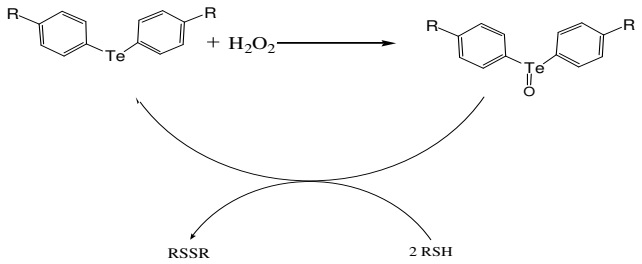
$$2RSeSG + 2GSH \rightarrow RSeSeR \qquad (8)$$

When the diselenide bond of diphenyl diselenide is disrupted, two selenols (reaction 4, and 5) can be yielded, differently from ebselen (Scheme-1, only one selenol molecule (3)), improving the catalytic reaction that is of particular significance to living cells. These mechanisms support our results that indeed, diphenyl diselenide has higher anti-oxidant potential than ebselen which we propose to be because of the difference in their catalytic cycle i.e. mode of formation of selenol/s.

Traces of iron salts are present in all biological systems, and any increase in the normal concentration will potentiate the toxic effects of oxygen. Therefore, much experimental attention has been given to analyze the role of iron, especially Fe^{2+}, in metal-mediated lipid peroxidation, and demonstrated that oxidation of Fe^{2+} to Fe^{3+} is closely linked to the onset

of the peroxidation process. In this respect, Several investigators have proposed some iron-oxygen complexes such as the ferryl ion [63], perferryl ion, [64] and Fe^{+2}-$O_2^{\bullet}$-Fe^{+3} complex [65], for oxidizing species of Fe (II) which can initiate or potentiate the extent of lipid peroxidation process by producing reactive oxygen species. A plausible mechanism by which DPDS is conferring protective action against Fe (II) induced lipid peroxidation in these homogenates is that DPDS could not only be operating as a free radical scavenger but may be interacting with Fe(II) or its oxidized forms.

We have also showed that DPDT significant protected tissue homogenate against Fe(II) induced lipid peroxidation. The anti-oxidant potency of DPDT can be explained by the fact that organotellurium compounds are readily oxidized from the divalent to the tetravalent state. This property makes them attractive as scavengers of reactive oxidizing agents such as hydrogen peroxide, hypochlorite, and peroxyl radicals, and as inhibitors of lipid peroxidation in chemical and biological systems [66]. This study also suggests that diorganoyl ditelluride are more reactive than structurally related diorganoyl diselenide compounds. The higher potency of DPDT compared with diphenyl diselenide and ebselen can be explained, essentially due to their higher electro negativity in relation to carbon associated with a larger atomic volume of the tellurium atom. Based on mechanistic studies, diaryl tellurides were pointed to exert an antioxidative effect by deactivating both peroxides and peroxyl radicals under the formation of telluroxides (Scheme 2). [67], and we may assume that DPDT may also work in a catalytic way and may lead to the formation of a intermediate telluroxide capable of quenching the hazardous effects of H_2O_2.



(Scheme 2)

We also tried to explore the possibilities if change in pH could modulate anti-oxidant of these organochalcogens, as based on the redox potential and electrochemical studied on organochalcogens, we worked on the hypothesis that the formation of stables selenolate (Se^{-1}) and tellurate (Te^{-1}) ions can increase the reducing properties of these moieties on the organochalcogenides and hypothetically can increase their antioxidant properties [68]. However contrary to our expectations we did not find ant alteration in the anti-oxidant status/potencies of the above tested compounds (data not shown). But, these studies confirm for the very first time that DPDS and DPDT and their reduced forms are redox active within patho- physiologically relevant potential range and can protect tissue from peroxidration. Although the observation in the present study cannot be directly related to in vivo conditions, it seems that the results may give us a clue to understand the role of Fe (II) in the iron-mediated cell injury and/or diseases under acidic conditions and a possible anti-oxidant effect of DPDS and DPDT in low pH mediated pathological conditions i.e. ischemic/reperfusion injury.

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7.0 Annexes

Drug and Chemical Toxicology, 2009; 00(00): 000–000

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RESEARCH ARTICLE

Low pH does not modulate antioxidant status of diphenyl ditelluride but exacerbates Fe (II)-induced lipid peroxidation in liver preparation

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Abstract
The relationship of acidosis and lipid peroxidation in liver homogenate was studied and the effect of pH on the antioxidant potential of diphenyl ditelluride is reported. Low pH increased the rate of lipid peroxidation both in the absence and presence of Fe (II), while diphenyl ditelluride (DPDT) inhibited the rate of lipid peroxidation in a concentration-dependent manner at all studied pH values. However, the change in pH did not alter the antioxidant activity of the compound. This study shows acidosis catalyzed oxidative stress in liver homogenate and the antioxidant potential of diphenyl ditelluride.

Keywords: *pH; Lipid peroxidation; Diphenyl ditelluride*

Introduction

Organotellurium compounds exhibit potent antioxidant activity mediated by their glutathione peroxidase (GPx) mimetic properties (Andersson et al., 1993; Engman et al., 1995). Of particular importance, our research group has obtained evidences indicating that diphenyl ditelluride (DPDT) causes marked neurotoxic effects in mice after acute or prolonged exposure either by subcutaneous or intraperitoneal routes (Nogueira et al., 2004). DPDT affects a number of neuronal processes and modifies the functionality of the glutamatergic system *in vitro* and *in vivo* (Nogueira et al., 2004), as well as inhibits the cerebral Na^+/K^+ATPase activity (Borges et al., 2005). Further, DPDT is highly toxic to rats and changes brain neurochemical parameters after *in vitro* and *in vivo* exposure (Moretto et al., 2005a, 2005b; Nogueira et al., 2004).

Recently, the possible pharmacological properties of organotellurium compounds have been investigated. Sredni et al. described that a tellurate, coded as AS-101, showed immunomodulating properties (Sredni et al., 1987). The toxicity of organotellurium compounds is mediated, at least in part, by their ability to react with thiols groups from biologically important molecules. In fact, organotellurium compounds, including DPDT, can inhibit thiol-containing enzymes, such as _-ALA-D (Barbosa et al., 1998; Maciel et al., 2000; Nogueira et al., 2001; Borges et al., 2003), Na+,K+ ATPase, (Borges et al., 2005), catepsin B (Cunha et al., 2005), and squalene monooxigenase (Laden and Porter, 2001). The inhibition of squalene monooxigenase can contribute to demyelination and, consequently, to neurotoxic effects of organotellurium compounds (Goodrum, 1998; Toews et al., 1991).

Based on the redox potential and electrochemical studies on organotellurium compounds (Collins et al., 2005), we worked on the hypothesis that the formation of stable tellurate (Te^{-1}) ions can increase the reducing properties of these moieties on the organochalcogenides (i.e., the organic compounds of the heavier chalcogens, in this case, the tellurides) and, hypothetically, can increase their antioxidant properties. However, there are no data in the literature supporting this assumption. This work work was designed to study different pH values (*in vitro*) and the influence of change

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in pH on the antioxidant effect of DPDT. Specifically, in this article, we studied the effect of pH on the antioxidant activity of DPDT in liver homogenate.

It has been suggested that metabolic changes induced by ischemia, such as acidosis, lead to intracellular iron delocalization, providing a source of iron in a form capable of catalyzing free radical production. Iron can stimulate free radical production in various ways: 1) via the breakdown of preexisting lipid hydroperoxides (ROOH) present in tissues to form lipid alkoxyl radical (RO.); 2) by entering a Fenton-type reaction to give OH. radical; and 3) via formation of iron-oxygen complexes, such as perferryl ions or ferrousdioxygen-ferric complexes (Braughler et al., 1986), which can substitute for OH. in the initiation of lipid peroxidation. Thus, the fall in intracellular pH that is associated with ischemia cannot only influence metabolic processes, but it can potentiate or act synergistically with oxidative stress, contributing to increased cellular injury (Balla et al., 1990). Solubility of iron increases at lower pH values; therefore, we hypothesized that decreasing the pH of the reaction medium will lead to increased lipid peroxidation. To this end, the influence of pH on either basal or Fe (II)-induced lipid peroxidation in rat-liver homogenate was also investigated in commonly used phosphate buffer.

Methodology

Preparation of buffers

All buffers (i.e., phosphate) were prepared at room temperature with constant ionic strength. Buffer solutions were maintained at 4°C until the initiation of the experiment. The pH was checked by a pH meter both in the start, middle, and final stages of the reaction. The temperature of the incubation medium was 37°C. Direct measurement of pH values in the tubes at higher temperature (i.e., 37°C) verified that actual pH values were typically within ±0.05. The final buffer concentration in all experiments was 10 mM.

Preparation of solutions

Fe (II) was freshly prepared by dissolving Fe (II) salt (i.e., ferrous sulfate; $Fe(SO_4)$) in water; the solution was prepared in the dark to avoid any photo-oxidation of the salt. The solution was prepared and immediately employed in the reaction. The minimum optimum concentration of iron to induce consistent lipid peroxidation was found to be 20 μM. In our study, we measured the prooxidant effect of iron at different pH values, ranging from physiological to acidic (i.e., 7.4–5.5), to get an insight about the role of iron at different pathophysiological conditions.

DPDT is unstable and sensitive. We prepared the solution freshly at the beginning of the experiment. The compound was dissolved in dimethyl sulfoxide (DMSO) and quickly added to the reaction mixture at the concentrations shown in Table 1. The compounds, first Fe (II) and then DPDT, were added quickly and simultaneously. A control (absent Fe (II)) is marked as the basal group in Table 1.

Animals and lipid peroxidation assay

For this study, 5–7 adult male Wistar rats from our own breeding colony (250–350 g) were used. Adult male Wistar rats were anesthetized with ether and

Table 1. Effect of diphenyl ditelluride (DPDT) on basal or Fe (II)-induced (20 μM) TBARS production in rat-liver homogenate at different pH levels.

	pH				
Treatment	5.4	5.8	6.4	6.8	7.4
Basal(B)[a]	1,243 ± 142	1,098 ± 101	912 ± 101	797 ± 89	545 ± 39
B + 0.4 μM[b]	899 ± 111	745 ± 132	563 ± 121	354 ± 37	283 ± 79
B + 1.0 μM[b]	1,098 ± 102	841 ± 112	681 ± 122	578 ± 67	354 ± 54
B + 4.0 μM[b]	913 ± 98	734 ± 98	539 ± 98	302 ± 23	281 ± 36
B + 10.0 μM[b]	999 ± 78	912 ± 119	613 ± 78	435 ± 29	302 ± 29
B + 40.0 μM[b]	1,014 ± 75	875 ± 120	733 ± 85	512 ± 54	332 ± 19
Fe(II)induced[a,*]	1,445 ± 134	1,254 ± 154	1,109 ± 121	998 ± 87	878 ± 76
Fe(II) + 0.4 μM[b]	873 ± 29	703 ± 29	619 ± 29	698 ± 29	529 ± 67
Fe(II) + 1.0 μM[b]	299 ± 37	245 ± 78	593 ± 19	539 ± 62	187 ± 54
Fe(II) + 4.0 μM[b]	254 ± 29	259 ± 54	212 ± 22	178 ± 45	152 ± 42
Fe(II) + 10.0 μM[b]	234 ± 17	199 ± 29	202 ± 34	154 ± 14	103 ± 39
Fe(II) + 40.0 μM[b]	243 ± 35	178 ± 45	187 ± 45	167 ± 19	114 ± 24

The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± standard error of the mean (n = 5–7). Different letters show a significant difference from the basal group and asterisk shows a significant main effect of Fe (II) at $P < 0.05$.

in pH on the antioxidant effect of DPDT. Specifically, in this article, we studied the effect of pH on the antioxidant activity of DPDT in liver homogenate.

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All buffers (i.e., phosphate) were prepared at room temperature with constant ionic strength. Buffer solutions were maintained at 4°C until the initiation of the experiment. The pH was checked by a pH meter both in the start, middle, and final stages of the reaction. The temperature of the incubation medium was 37°C. Direct measurement of pH values in the tubes at higher temperature (i.e., 37°C) verified that actual pH values were typically within ±0.05. The final buffer concentration in all experiments was 10 mM.

Preparation of solutions

Fe (II) was freshly prepared by dissolving Fe (II) salt (i.e., ferrous sulfate; $Fe(SO_4)$) in water; the solution was prepared in the dark to avoid any photo-oxidation of the salt. The solution was prepared and immediately employed in the reaction. The minimum optimum concentration of iron to induce consistent lipid peroxidation was found to be 20 μM. In our study, we measured the prooxidant effect of iron at different pH values, ranging from physiological to acidic (i.e., 7.4–5.5), to get an insight about the role of iron at different pathophysiological conditions.

DPDT is unstable and sensitive. We prepared the solution freshly at the beginning of the experiment. The compound was dissolved in dimethyl sulfoxide (DMSO) and quickly added to the reaction mixture at the concentrations shown in Table 1. The compounds, first Fe (II) and then DPDT, were added quickly and simultaneously. A control (absent Fe (II)) is marked as the basal group in Table 1.

Animals and lipid peroxidation assay

For this study, 5–7 adult male Wistar rats from our own breeding colony (250–350 g) were used. Adult male Wistar rats were anesthetized with ether and

Table 1. Effect of diphenyl ditelluride (DPDT) on basal or Fe (II)-induced (20 μM) TBARS production in rat-liver homogenate at different pH levels.

	pH				
Treatment	5.4	5.8	6.4	6.8	7.4
Basal(B)[a]	1,243 ± 142	1,098 ± 101	912 ± 101	797 ± 89	545 ± 39
B + 0.4 μM[b]	899 ± 111	745 ± 132	563 ± 121	354 ± 37	283 ± 79
B + 1.0 μM[b]	1,098 ± 102	841 ± 112	681 ± 122	578 ± 67	354 ± 54
B + 4.0 μM[b]	913 ± 98	734 ± 98	539 ± 98	302 ± 23	281 ± 36
B + 10.0 μM[b]	999 ± 78	912 ± 119	613 ± 78	435 ± 29	302 ± 29
B + 40.0 μM[b]	1,014 ± 75	875 ± 120	733 ± 85	512 ± 54	332 ± 19
Fe(II)induced[a,*]	1,445 ± 134	1,254 ± 154	1,109 ± 121	998 ± 87	878 ± 76
Fe(II) + 0.4 μM[b]	873 ± 29	703 ± 29	619 ± 29	698 ± 29	529 ± 67
Fe(II) + 1.0 μM[b]	299 ± 37	245 ± 78	593 ± 19	539 ± 62	187 ± 54
Fe(II) + 4.0 μM[b]	254 ± 29	259 ± 54	212 ± 22	178 ± 45	152 ± 42
Fe(II) + 10.0 μM[b]	234 ± 17	199 ± 29	202 ± 34	154 ± 14	103 ± 39
Fe(II) + 40.0 μM[b]	243 ± 35	178 ± 45	187 ± 45	167 ± 19	114 ± 24

The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± standard error of the mean ($n = 5–7$). Different letters show a significant difference from the basal group and asterisk shows a significant main effect of Fe (II) at $P < 0.05$.

pH of the media. The possibility of reduction of Fe (III) to Fe (II) by interaction with $O_2^{\bullet -1}$ at the early phase of lipid peroxidation under acidic conditions, perhaps via an intermediate, perferryl iron (Pederson et al., 1973), cannot be excluded (Equations 5 and 6):

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -1} \quad (5)$$

$$Fe^{3+} + O_2^{\bullet -1} \rightarrow \left[Fe^{3+} — O_2^{\bullet -1} \leftrightarrow Fe^{2+} — O_2\right] \leftrightarrow Fe^{2+} + O_2 \quad (6)$$

The pH environment and a series of chain reactions seem to provide optimal conditions for maximal catalytic efficiency of iron. Thus, the acidic pH not only releases iron from a "safe" site (Balla et al., 1990), but also potentiates the prooxidant effect of Fe (II), as we observed from the significant increase in TBARS production at pH (6.8–5.4) (Figure 1). These observations are in agreement with our previous reports that, indeed, low pH enhances iron-mediated lipid peroxidation in rat-tissue homogenate as well phospholipids liposomes from egg yolk (Hassan et al., 2009).

We have evaluated the inhibitory effect of DPDT against iron-mediated lipid peroxidation at a range of pH values. A curve of concentration was performed in this regard. DPDT significantly ($P < 0.05$) reduced Fe (II)-induced TBARS at all studied pH values in liver homogenate (Table 1). Being a very potent antioxidant compound, the IC_{50} value found was less than 1.00 μM (for Fe (II)-induced TBARS, at all studied pH values) after testing the following concentrations (i.e., 0.4, 1.0, 4.0, 10.0, and 40.0 μM), as shown in Table 1.

The antioxidant potency of DPDT can be explained by the fact that organotellurium compounds are readily oxidized from the divalent to the tetravalent state. This property makes them attractive as scavengers of reactive oxidizing agents, such as hydrogen peroxide, hypochlorite, and peroxyl radicals, and as inhibitors of lipid peroxidation in chemical and biological systems (Andersson et al., 1993, 1994). Despite the fact that DPDT has low redox potential (Collins et. al., 2005), and the reduced form was expected to be a good reducing agent, but we did not find any alteration in the antioxidant potential of DPDT with respect to pH value.

Conclusions

We have shown that acidic pH enhances the detrimental effect of iron in liver preparation. In the present work, we demonstrated that DPDT can protect Fe (II)-induced TBARS production in liver homogenates at all studied pH values. The implication from this work, for a biological system, is that DPDT is a redox-active compound and can work with the same potency under various physiological conditions. For the possible potential therapeutic use of DPDT, however, one should consider that it is also highly neurotoxic (Meotti et al., 2003; Sailer et al., 2004).

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***Declaration of interest*:**

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Towards the mechanism and comparative effect of diphenyl diselenide, diphenyl ditelluride and ebselen under various pathophysiological conditions in rat's kidney preparation

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ABSTRACT

As an extension of our previous work we not only evaluated the relationship between acidosis and lipid peroxidation in rat's kidney homogenate, but also determined for the first time the potential anti-oxidant activity of diphenyl diselenide, diphenyl ditelluride and ebselen at a range of pH values (7.4–5.4). Because of the pH dependency of iron redox cycling, pH and iron need to be well controlled and for the reason we tested a number of pH values (from 7.4 to 5.4) to get a closer idea about the role of iron under various pathological conditions. Acidosis increased rate of lipid peroxidation in the absence Fe (II) in kidney homogenates especially at pH 5.4. This higher extent of lipid peroxidation can be explained by; the mobilized iron which may come from reserves where it is weakly bound. Addition of iron (Fe) chelator desferoxamine (DFO) to reaction medium completely inhibited the peroxidation processes at all studied pH values including acidic values (5.8–5.4). In the presence of Fe (II) acidosis also enhanced detrimental effect of Fe (II) especially at pH (6.4–5.4). Diphenyl diselenide significantly protected lipid peroxidation at all studied pH values, while ebselen offered only a small statistically non-significant protection. The highest anti-oxidant potency was observed for diphenyl ditelluride. These differences in potencies were explained by the mode of action of these compounds using their catalytic anti-oxidant cycles. However, changing the pH of the reaction medium did not alter the anti-oxidant activity of the tested compounds. This study provides evidence for acidosis catalyzed oxidative stress in kidney homogenate and for the first time anti-oxidant potential of diphenyl diselenide and diphenyl ditelluride not only at physiological pH but also at a range of acidic values.

1. Introduction

Numerous human diseases, ranging from Rheumatoid Arthritis to neurodegenerative diseases, are associated with an imbalance in cellular redox systems, a biochemical condition known as Oxidative Stress (OS) [1–3]. The use of either natural or synthetic anti-oxidants is therefore increasingly considered in medicine to target OS related disorders. The broad spectrum of anti-oxidants divides into different classes, among which catalytic anti-oxidants, such as enzymes and enzyme mimics, play an important role [4] Unlike 'one shot' anti-oxidants, such catalysts are able to counteract OS at low concentrations, and are also sensitive towards the presence of their stressor (and reducing) substrates, hence endowing them with a certain degree of selectivity [5,6]. Catalysts mimicking the activity of the human enzyme glutathione peroxidase (GPx) are particularly interesting [7].

Organoselenium compounds exhibit potent anti-oxidant activity mediated by their glutathione peroxidase mimetic properties. In line with this, ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one), a synthetic selenium-containing heterocycle has been used experimentally and clinically with success in a variety of situations where free radicals are involved. Diphenyl Diselenide (DPDS), the simplest diaryl diselenide, is particularly important as a potential anti-oxidant drug in view to the fact that it has been shown to be more active as a glutathione peroxidase mimic, less toxic to rodents than ebselen and has also low toxicity for non-rodent mammals after long term exposure [8].

Similarly, there has been a considerable interest in organotellurium compounds as potential anti-oxidants in living systems against several pro-oxidant agents, such as hydrogen peroxide, peroxynitrite, hydroxyl radicals and superoxide radical anion [9–14], since these compounds may mimic glutathione peroxidase activity [15,11]. This property is thought to be due to oxidation of Te from the divalent to the tetravalent state. Besides, tellurides are promising antitumoral drugs and their chemoprotective effects can be related to their cytotoxic properties and to their ability to inhibit

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important enzymes necessary for the tumor growth [16–18]. These pharmacological properties are much more evident in organotellurium compounds than in selenium or sulfur analogues, making these compounds extremely attractive in medical therapies.

However there is scarcity of data in the literature about the comparative effect of DPDS, diphenyl ditelluride (DPDT) and ebselen under various pathophysiological conditions. The work was designed in this regard to get a different pH media (in vitro) and study the extent of Fe (II) induced lipid peroxidation and anti-oxidant potential of these organochalcogens compounds. For the purpose influence of pH on (a) either basal or Fe (II) induced lipid peroxidation in rat kidney homogenates, and (b) on anti-oxidant potential of DPDS, DPDT and ebselen was investigated.

2. Materials and methods

2.1. Organochalcogens synthesis and preparation

DPDS, DPDT and ebselen were synthesized according to literature method [19–21] and were dissolved in DMSO. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed that the compounds obtained presented analytical and spectroscopic data in full agreement with their assigned structures. The chemical purity of compounds (99.9%) was determined by GC/HPLC. Solutions of compounds were prepared in DMSO few minutes (5–10 min) before the experiments. The solution is stable for the period utilized in the experimental protocols performed in this study. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Animals

Adult male Wistar rats from our own breeding colony (250–350 g) were maintained in an air-conditioned room (22–25 °C) under natural lighting conditions, with water and food (Guabi, RS, Brazil) ad libitum. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil.

2.3. Tissue preparation

Animals were anesthetized with ether and killed by decapitation. The kidneys were quickly removed, placed on ice, and homogenized within 10 min, in 10 mmol/l Tris/HCl buffer, pH 7.4 (in 10 volume). The homogenates were centrifuged at 4000 × g at 4 °C for 10 min to yield a low speed supernatant fraction (S1) that was used immediately for TBARS assay [22].

2.4. Lipid peroxidation assay

All buffers were prepared at room temperature with constant ionic strength. Buffer solutions were maintained at 4 °C until the initiation of the experiment. Direct measurement of pH values in the tubes at higher temperature i.e. (37 °C) verified that actual pH values were typically within ±0.05. The final buffer concentration in all experiments was 10 mM.

Fe (II) was freshly prepared by dissolving Fe (II) salt i.e. ferrous sulfate {($Fe(SO_4)$)} in water, the solution was prepared in dark to avoid any photo-oxidation of the salt. The solution was prepared and immediately employed in reaction. The minimum optimum concentration of iron to induce consistent lipid peroxidation was found to be 20 μM. In our study we have measured the pro-oxidant effect of iron at different pH ranging from physiological to acidic ones i.e. 7.4–5.5, to get an insight about role of iron at different pathophysiological conditions.

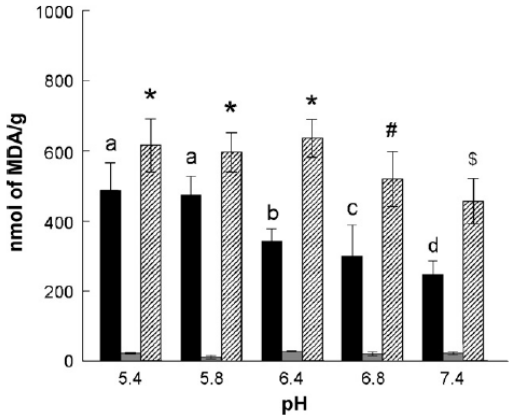

Fig. 1. Effect of pH on basal (black shaded bar) and Fe (II)-induced (bar with lines) (20 μM) TBARS production in supernatants of homogenates from kidney of rats. TBARS are expressed as nmol of MDA. Data are expressed as means ± S.E.M. (n = 5–7). Main significant effect of Fe (II) was found at all pH values. Letters indicate significant effect of pH in the absence of iron (black shaded bar) while; symbols indicate significant effect of pH in the presence of iron (bar with lines) at $p < 0.05$. Gray shaded bars show significant inhibitory effect of desferoxamine (DFO) (1 mM) at all studies pH values. Bars that do not share the same letter or symbol are different from each other at $p < 0.05$ by Duncan's multiple tests.

DPDS, DPDT and ebselen were prepared freshly i.e. just at the beginning of the experiment. The compounds were dissolved in DMSO and quickly added to the reaction mixture at the concentrations mentioned in (Figs. 1–4). The compounds i.e. Fe (II) and then organochalcogens were added simultaneously without loosing time. It should be worthwhile to note that we also run a control that is in the absence of Fe (II) and is marked as basal group in (Figs. 2–4).

Lipid peroxidation was determined by measuring thiobarbituric acid-reactive species (TBARS) as described by [22] with minor modifications [23]. Kidney homogenate was prepared by homogenization as described above. Aliquots of the homogenate (100 μl) was incubated for 60 min in a medium containing 10 mM sodium phosphate buffer of different pH ranging from 5.4 to 7.4 (pH was checked after the addition of kidney supernatant both at the beginning and at the end of the incubation period) and in the presence of other reagents at concentrations indicated in the legends. The mixtures were incubated at 37 °C for 60 min. The reaction was stopped by addition of 0.5 ml of acetic acid buffer and lipid peroxidation products were measured by the addition of 0.5 ml of TBA 0.6% and 0.2 ml of SDS 8.1%. The color reaction was developed by incubating tubes in boiling water for 60 min. TBARS levels were measured at 532 nm using a standard curve of MDA. The values are expressed in nmol MDA/g of tissue. Where indicated, solutions of $FeSO_4$ were made just before use in distilled water.

2.5. Statistical analysis

The results are expressed as the mean ± standard error (S.E.M.). Data were analyzed statistically by analysis of variance i.e. (two- and three-way ANOVA), followed by univariate analysis and Duncan's multiple range test when appropriate.

3. Results

3.1. Effect of pH on basal and or Fe (II)-induced TBARS production in kidney homogenates

Two-way ANOVA i.e. (5 (pHs)) × 2 (basal/iron) of renal TBARS production indicated a significant main effect of pH and Fe (II)

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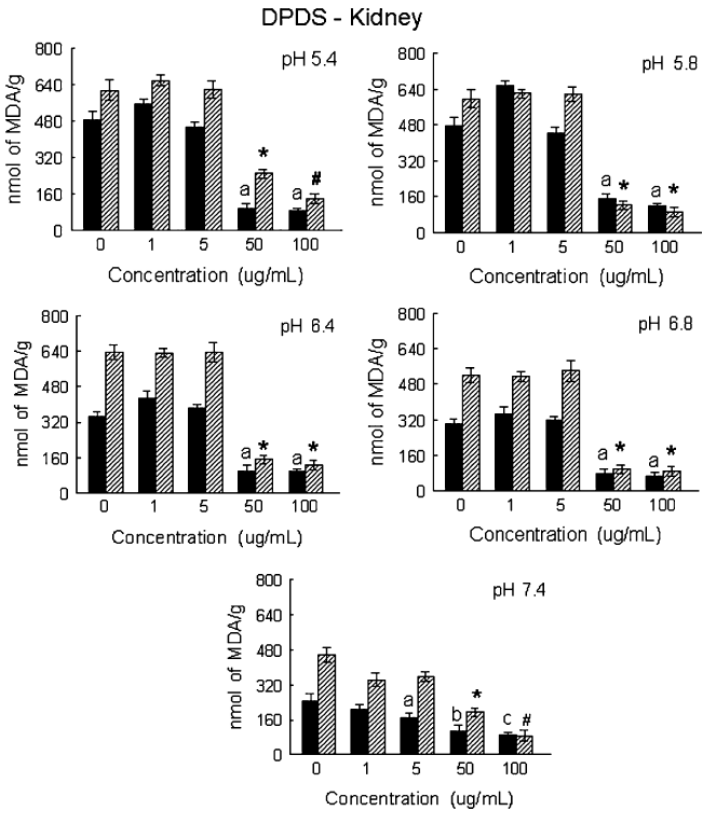

Fig. 2. Effect of DPDS on basal (shaded bar) or Fe (II)-induced (bar with lines) TBARS production in rat's kidney homogenate at different pH. Data are expressed as means ± S.E.M. (n = 5–7). Fe (II) caused a significant effect on TBARS production at all studied pH values at basal concentration. Letters indicate significant effect of DPDS in the absence of iron (black shaded bar); while symbols indicate significant effect of DPDS in the presence of iron (bar with lines) as compared to basal values at $p < 0.05$. Bars that do not share the same letter or symbol are different from each other at $p < 0.05$ by Duncan's multiple tests.

and also a significant pH × Fe (II) interaction ($p < 0.05$). Basal and Fe (II)–induced TBARS production increased significantly as the pH decreased from 7.4 to 5.4 as shown in (Fig. 1).

3.2. Effect of DPDS, DPDT and ebselen on basal and or Fe (II)-induced TBARS production in kidney homogenates

3.2.1. Effect of DPDS

Three-way ANOVA of TBARS production i.e. 5 pHs × 5 concentrations of (DPDS) × 2 (basal/iron) revealed a significant main effect of pH, DPDS and Fe (II) and also significant pH × Fe (II) × DPDS interaction ($p < 0.05$). It is possible to observe that Fe (II) caused statistically significant increase in TBARS production while DPDS reduced it in kidney homogenate (Fig. 2).

3.2.2. Effect of DPDT

Similarly, three-way ANOVA of TBARS production i.e. 5 pHs × 5 concentrations of (DPDT) × 2 (basal/iron) revealed a significant main effect of pH, DPDT and Fe (II) and also significant pH × Fe (II) × DPDT interaction ($p < 0.05$). It is possible to observe that DPDT reduced TBARS production in kidney homogenate as shown in (Fig. 3).

3.2.3. Effect of ebselen

While, two-way ANOVA i.e. (pH × basal/iron) for TBARS production in case of ebselen revealed a significant main effect of Fe (II) and at all studied pH values ($p < 0.05$). It is apparent from (Fig. 4) that ebselen did not offer any protective effect on basal and iron-induced lipid peroxidation at all stated pH values in kidney homogenates.

4. Discussion

4.1. Why low pH increased extent of lipid peroxidation?

There are several biochemical/pathological situations where pH of the system can go as low as 4.5, which can influence the role of iron under these conditions. Iron-catalyzed lipid peroxidation in K-562 cells was shown to be pH-dependent, the lower the extracellular pH (decreasing from 7.5 to 5.5), the higher the free radical flux; the lower the pH, the greater the membrane permeability of iron [24]. In the phagolysosomes of cells where pH is around 5.5 [25], this pH environment seems to provide optimal conditions for maximal catalytic efficiency and solubility of iron [26,27]. In comparison with normal tissue, human tumors have relative low pH levels [28]. These low levels in tumors may increase iron released from ferritin and lactoferrin and enhance oxidative damage as well. It should be worthwhile to note that during ischemia the pH of the tissue can reach as low as 6.0. Similarly the pH determined in different cellular compartments is in the range of about 5.0–7.5 [29]. It is also well known that lysosomes are acidic, having a pH of about 4.7–4.8 in macrophages [30].

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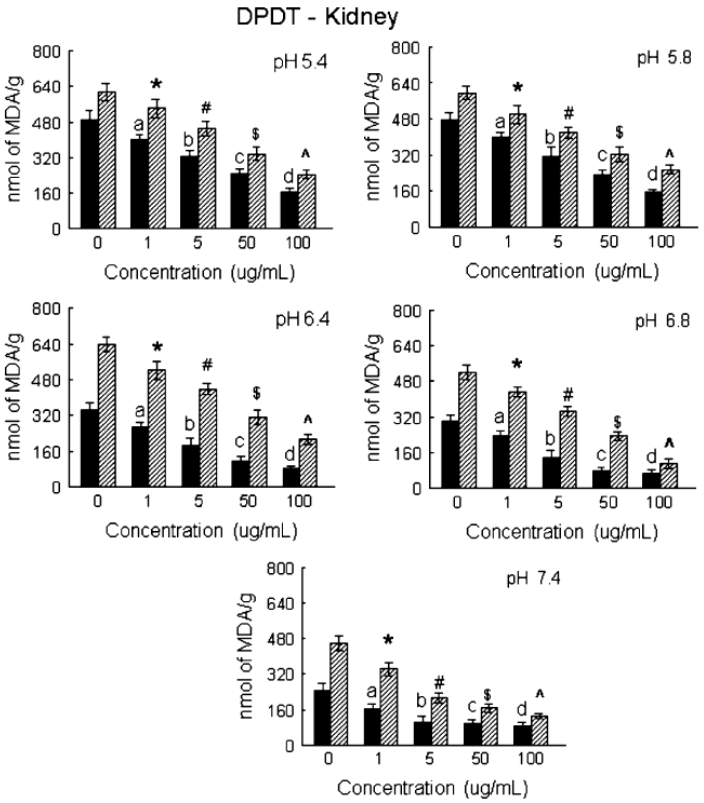

Fig. 3. Effect of DPDT on basal (shaded bar) or Fe (II)-induced (bar with lines) TBARS production in rat's kidney homogenate at different pH. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n = 5–7). Fe (II) caused a significant effect on TBARS production at all studied pH values at basal concentration. Letters indicate significant effect of DPDT in the absence of iron (black shaded bar) while; symbols indicate significant effect of DPDT in the presence of iron (bar with lines) as compared to basal values at $p < 0.05$. Bars that do not share the same letter or symbol are different from each other at $p < 0.05$ by Duncan's multiple tests.

Most studies (but not all) have shown that acidity increases the oxidation of lipids. The autoxidation of linoleic acid was increased by acidity (whereas that of methyl linoleate was decreased by acidity) [31] and the oxidation of polyunsaturated fatty acids by iron–ascorbate, by ferritin–ascorbate or by tissue homogenates [32] was much faster at acidic pH. The oxidation of brain homogenates [33] or brain slices [34] was increased greatly at acidic pH. Phospholipid liposomes are oxidized faster at acidic pH by human activated neutrophils [35] or by autoxidation [36]. In contrast, the autoxidation of phospholipid emulsions has been reported to be slower at acidic pH [37].

Aliquots (100 μl) of kidney homogenate were incubated for 60 min in a medium contains 10 mM sodium phosphate buffer, of different pH ranging from 5.4 to 7.8. It is apparent from (Fig. 1) that the rate of per oxidation exhibits a dramatic increase as the pH is decreased from 7.8 to 5.4 both in the presence and absence of Fe (II) in kidney homogenates. The enhancement of pH-dependent lipid peroxidation can be attributed to mobilized iron which may come from reserves where it is weakly bound. The pH dependency of TBARS production can be explained by the fact, firstly, that the protein transferrin carries two iron ions, although it is normally only about one third saturated with iron [38]. Transferrin loses its bound iron at acidic pH. The pH-dependent affinity of transferrin for iron decreases under acidic conditions leading to dissociation of iron from transferrin and other proteins like ferritin and lactoferrin [39].

The mobilized iron Fe (II) can interact with enzymatically and/or non-enzymatically generated superoxide ($O_2^{\bullet -1}$) (Haber–Weiss reaction) and/or hydrogen peroxide (H_2O_2) (Fenton reaction) [40] producing reactive oxygen species.

In fact, $O_2^{\bullet -1}$ may be produced directly from dissolved oxygen (O_2) in aqueous media in the Fe^{2+}-mediated basal/autoxidation reactions as follows:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -1} \quad (1)$$

The superoxide produced in above reaction (1) may react with Fe (II) in a metal catalyzed (Haber–Weiss reaction) reaction producing hydroperoxides reaction (2). Which further reaction with Fe (II) produces the extremely reactive hydroxyl radical reaction (3), which may then abstract hydrogen atoms from polyunsaturated fatty acids.

$$Fe^{2+} + O_2^{\bullet -1} + 2H^+ \rightarrow Fe^{3+} + H_2O_2 \quad (2)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-1} + OH^{\bullet} \quad (3)$$

Indeed, transferrin has been shown to release bound iron during in vitro acidotic conditions, particularly as pH falls to <6.0 and brain cortical homogenates media release iron under acidic conditions [39]. Furthermore, acidosis also increases TBARS in phospholipids liposomes and brain homogenate and [41].

To support the mechanism and to explore the involvement of Fe (II) especially at low pH, we added iron chelator i.e. desferoxamine,

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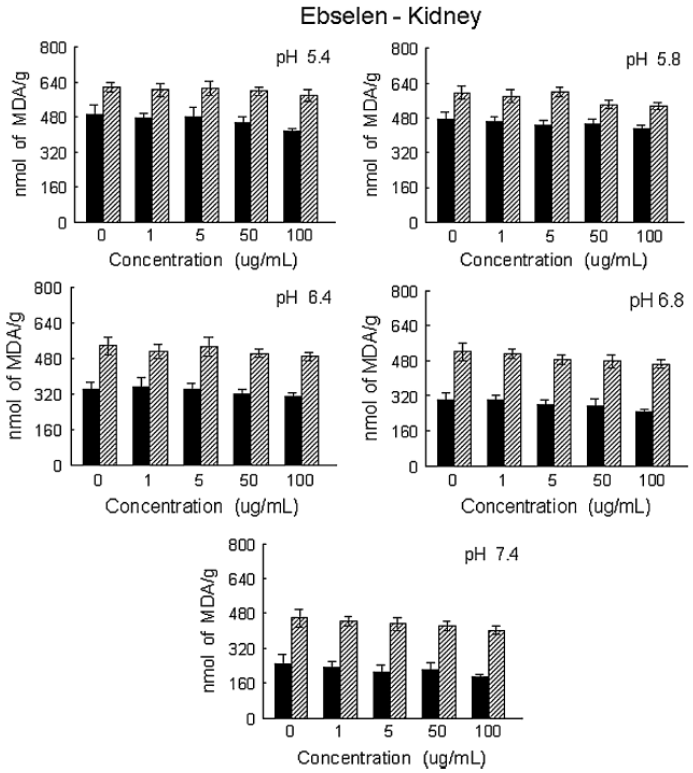

Fig. 4. Effect of ebselen on basal (shaded bar) or Fe (II)-induced (bar with lines) TBARS production in rat's kidney homogenate at different pH. The values are expressed as nmol of MDA per gram of tissue. Data are expressed as means ± S.E.M. (n = 5–7). Fe (II) caused a significant effect on TBARS production at all studied pH values at basal concentration. Ebselen did not offer any protection against MDA production at none of the pH values.

DFO (1 mM) in the absence of extracellular added iron. The addition of DFO significantly inhibited TBARS production at all studied pH values indicating the Fe (II) release (especially at acidic pH values) and its participation in lipid peroxidation processes as shown in (Fig. 1). The data presented here provides direct evidence for enhanced lipid peroxidation in kidney homogenates at acidic pH which are consistent with our previous observations [42]. In the same way, acidic pH enhances the detrimental effect of iron. In our study we have addressed the catalytic efficiency of iron in kidney homogenates as the environmental pH changes. Thus, acidic pH not only releases iron from "safe" sites, but also potentiate the pro-oxidant effect of Fe (II) as apparent (Fig. 1) from increased lipid peroxidation.

4.2. Differences in activities of organochalcogens

DPDS significantly reduced both basal and Fe (II) induced TBARS at all studied pH values in kidney homogenates as shown in (Fig. 2). Surprisingly, ebselen did not offer any protection against TBARS production at all studied pH values in kidney preparation (Fig. 4).

To explain the difference between the observed activities of the these two organoselenium compounds, we can take help from literature in which the available information reveals a hypothetical catalytic cycle in which, the Se–N bond of ebselen (1) is readily cleaved by thiols to produce the corresponding selenenyl sulfides (2), which upon reduction by excess thiols produces selenol (3). Finally, the selenol reacts with organic hydroperoxide (produced in reaction 2) to form ebselen via ebselen-selenic acid (4) as shown in (Fig. 5) [43].

Similarly, for DPDS in the presence of excess thiol, the diselenide (RSeSeR (1)) is reduced to the selenolate RSe^{-1} (3) with the formation of RSSeR (2). Nucleophilic attack of thiol on RSSeR produces the disulfide and the 2nd selenolate RSe^{-1} ion (3). The two selenolate molecules (3) produced can react with H_2O_2 (produced in reac-

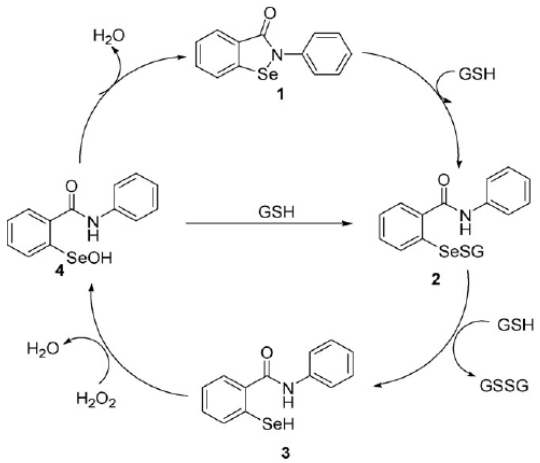

Fig. 5. Catalytic anti-oxidant cycle of ebselen.

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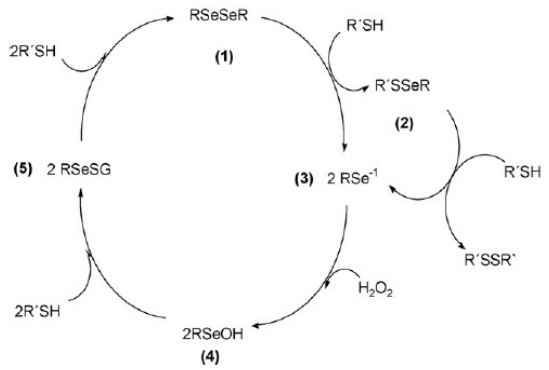

Fig. 6. Catalytic anti-oxidant cycle of diphenyl diselenide.

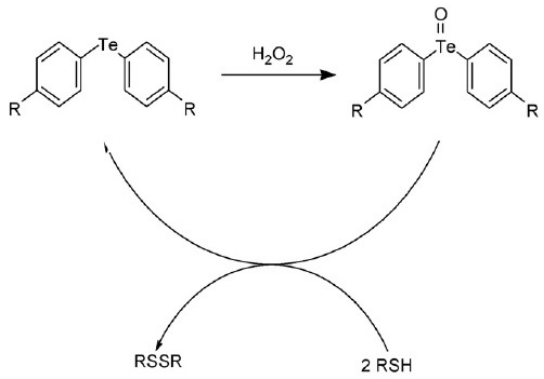

Fig. 7. Catalytic anti-oxidant cycle of diaryl telluride.

tion (2)) producing selenic acid (4) which upon reactions with RSH produces selenyl sulfide (5) and regenerate a molecule of diphenyl diselenide (1) as shown in (Fig. 6). When the diselenide bond of diphenyl diselenide is disrupted, two selenol (3) can be yielded, differently from ebselen (Fig. 5, only one selenol molecule (3)), improving the catalytic reaction that is of particular significance to living cells. These mechanisms support our results that indeed, diphenyl diselenide has higher anti-oxidant potential than ebselen which we propose to be because of the difference in their catalytic cycle i.e. mode of formation or the number of selenol molecules formed.

Traces of iron salts are present in all biological systems, and any increase in the normal concentration will potentiate the toxic effects of oxygen. Therefore, much experimental attention has been given to analyze the role of iron, especially Fe^{2+}, in metal-mediated lipid peroxidation, and demonstrated that oxidation of Fe^{2+} to Fe^{3+} is closely linked to the onset of the peroxidation process. In this respect, Several investigators have proposed some iron-oxygen complexes such as the ferryl ion [44], perferryl ion [45] and Fe^{+2}–O_2•–Fe^{+3} complex [46], for oxidizing species of Fe (II) which can initiate or potentiate the extent of lipid peroxidation process by producing reactive oxygen species. A plausible mechanism by which DPDS is conferring protective action against Fe (II) induced lipid peroxidation in these homogenates is that DPDS could not only be operating as a free radical scavenger but may also be interacting with Fe (II) or its oxidized forms.

We have also showed that DPDT significant protected kidney preparation against Fe (II) induced lipid peroxidation as shown in (Fig. 3). The anti-oxidant potency of DPDT can be explained by the fact that organotellurium compounds are readily oxidized from the divalent to the tetravalent state. This property makes them attractive as scavengers of reactive oxidizing agents such as hydrogen peroxide, hypochlorite, and peroxyl radicals, and as inhibitors of lipid peroxidation in chemical and biological systems [14]. This study also suggests that diorganoyl ditelluride are more reactive than structurally related diorganoyl diselenide compounds. The higher potency of DPDT compared with diphenyl diselenide and ebselen can be explained, essentially due to their higher electro negativity in relation to carbon associated with a larger atomic volume of the tellurium atom. Based on mechanistic studies, diaryl tellurides were pointed to exert an antioxidative effect by deactivating both peroxides and peroxyl radicals under the formation of telluroxides (Fig. 7) [47,48], and we may assume that DPDT may also work in a catalytic way and may lead to the formation of an intermediate telluroxide capable of quenching the hazardous effects of H_2O_2. We also tried to explore the possibilities if change in pH could modulate anti-oxidant of these organochalcogens, as based on the redox potential and electrochemical studied on organochalcogens, we worked on the hypothesis that the formation of stables selenolate (Se^{-1}) and tellurate (Te^{-1}) ions can increase the reducing properties of these moieties on the organochalcogenides and hypothetically can increase their anti-oxidant properties. However contrary to our expectations we did not find ant alteration in the anti-oxidant status/potencies of the above tested compounds. Although the observation in the present study cannot be directly related to in vivo conditions, it seems that the results may give us a clue to understand the role of Fe (II) in the iron-mediated cell injury and/or diseases under acidic conditions and a possible anti-oxidant effect of DPDS and DPDT in low pH mediated pathological conditions i.e. ischemic/reperfusion injury. But, these studies confirm for the very first time that DPDS and DPDT and their reduced forms are redox active within pathophysiologically relevant potential range and can protect tissue from peroxidation.

Conflict of interest

There is no conflict of interest in the conduct and reporting of research (e.g., financial interests in a test or procedure, funding by pharmaceutical companies for drug research).

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