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Abstract: Lipid hydroperoxides play an important role in various pathophysiological processes. Therefore, a simple model for organic hydroperoxides could be helpful to monitor the biologic effects of endogenous and exogenous compounds. The electron paramagnetic resonance (EPR) spin-trapping technique is a useful method to study superoxide (O$_2^•−$) and hydroxyl radicals. The aim of our work was to use EPR with the spin trap 5-tert-butoxycarbonyl-5-methyl-1-pyrroline-N-oxide (BMPO), which, by trapping O$_2^•−$ produces relatively stable •BMPO-OOH spin-adduct, a valuable model for organic hydroperoxides. We used this experimental setup to investigate the effects of selected sulfur/selenium compounds on •BMPO-OOH and to evaluate the antioxidant potential of these compounds. Second, using the simulation of time-dependent individual BMPO adducts in the experimental EPR spectra, the ratio of •BMPO-OH/•BMPO-OOH—which is proportional to the transformation/decomposition of •BMPO-OOH—was evaluated. The order of potency of the studied compounds to alter •BMPO-OOH concentration estimated from the time-dependent •BMPO-OH/•BMPO-OOH ratio was as follows: Na$_2$S$_4$ > Na$_2$S$_4$/SeO$_3^{2−}$ > H$_2$S/SeO$_3^{2−}$ > Na$_2$S$_2$ ~Na$_2$S$_2$/SeO$_3^{2−}$ ~H$_2$S > SeO$_3^{2−}$ ~SeO$_4^{2−}$ ~control. In conclusion, the presented approach of the EPR measurement of the time-dependent ratio of •BMPO-OH/•BMPO-OOH could be useful to study the impact of compounds to influence the transformation of •BMPO-OOH.

Keywords: hydroperoxides; antioxidants; EPR spectra simulation; •BMPO-OOH spin-adduct; superoxide; radical; hydrogen sulfide; polysulfides; selenite; DNA

1. Introduction

Exogenously added and endogenously produced hydrogen sulfide (H$_2$S) and polysulfides affect many physiological and pathologic processes [1–4]. They modulate oxidative stress by reacting with reactive oxygen and nitrogen species [1,5–7]. Selenium (Se) is an essential trace element for humans, with multiple and complex effects on health, having antioxidant properties due to its presence in 25 selenoproteins in the form of selenocysteine amino acid [8]. Se compounds and H$_2$S are
present in living organisms and either alone or in combination interact with reactive oxygen species (ROS) [1,9–12]. In our previous work, we found that products of the sulfide/selenite (H₂S/SeO₃²⁻) interaction scavenge superoxide-derived radicals, cleave plasmid DNA (pDNA) and modulate tonus of isolated rat aorta and blood pressure [13]. However in this work, using the procedure of addition of KO₂ as a source of (O₂•−) into the mixture of the compounds (H₂S, SeO₃²⁻) with the spin-trapping agent 5-tert-butoxycarbonyl-5-methyl-1-pyrroline N-oxide (BMPO) it was not clear which components of the electron paramagnetic resonance (EPR) spectra resulted from the compounds/O₂•− or compounds/BMPO-OOH interactions. To solve this issue, a new experimental strategy for the interaction of the compounds with •BMPO-OOH only is herein presented.

Lipid peroxidation is a process under which oxidants such as free radicals attack lipids containing carbon-carbon double bond(s), leading to the radical chain reactions. It has an important role in cell biology and in various pathophysiological processes in which lipid hydroperoxides display a crucial function [14]. Importantly, many pathophysiological states can be regulated by the modulation of lipid peroxidation induced by exogenous compounds. Therefore, simple models of organic hydroperoxide could be useful for studying the effects of various compounds. EPR spin-trapping technique using cyclic nitrone BMPO is a reliable method to study O₂•− and hydroxyl radicals [15–17].

The spin-trapping agent BMPO in aqueous solutions represents a racemic mixture of two enantiomers [S (−) and R (+)], and the previous detailed study evidenced that starting with racemate, as well as with the individual enantiomers, the reaction with O₂•− resulted in the identical EPR spectrum representing two signals of diastereoisomers at the same ratio (trans and cis with respect to the tert-butoxycarbonyl group) (Scheme 1). The EPR spectra of •BMPO-OOH diastereoisomers are characterized with the similar nitrogen splittings and slightly different β-hydrogen splittings [18–20]. Analogously, the experimental EPR spectra of •BMPO-OOH were interpreted considering the superposition of individual signals of two diastereoisomers with the different hyperfine coupling constants [15,21] (Scheme 1).

![Scheme 1. 5-tert-butoxycarbonyl-5-methyl-1-pyrroline-N-oxide (BMPO) spin trap enantiomers and illustration of the diastereoisomeric spin-adducts generation in BMPO reactions with O₂•− and HO•. Nuclei included in the simulation of the experimental EPR spectra of the •BMPO-OOH and •BMPO-OH adducts are marked in red.](image_url)

Therefore, the aim of our work was to use EPR with BMPO—which in the presence of O₂•− forms a relatively stable •BMPO-OOH and can serve as a model for organic hydroperoxide—to study the
effects of Na$_2$Sn ($n = 1, 2, 4$) and Na$_2$SeO$_n$ ($n = 3, 4$) on their own or their mixture Na$_2$Sn/Na$_2$SeO$_n$. This approach enables the comparison of the relative potential of the investigated compounds to affect the ROOH bond and to eliminate radicals formed during its decomposition. Since 10% DMSO (v/v) is used in the studied system, the procedure is useful for evaluating antioxidant potency of compounds insoluble in water.

2. Materials and Methods

2.1. Chemicals

Stock solutions of sodium selenite (Na$_2$SeO$_3$, 10 or 40 mmol L$^{-1}$, Sigma-Aldrich 214485, Saint Louis, MO, USA) and sodium selenate (Na$_2$SeO$_4$, 10 mmol L$^{-1}$, Sigma-Aldrich S0882, Saint Louis, MO, USA) were prepared freshly in deionized H$_2$O, stored at 23 °C and used within 5 h. Na$_2$SeO$_3$ dissolves in solution to yield mostly H$_2$SeO$_3$ at acidic pH, HSeO$_3^-$ at neutral pH and SeO$_2^{2-}$ at alkaline pH. For simplicity, the terms SeO$_2^{2-}$ and SeO$_4^{2-}$ are employed as representative expression to encompass the total mixture of different (de)protonation states. Spin-trapping agent 5-tert-butoxycarbonyl-5-methyl-1-pyrroline-N-oxide (BMPO, 100 mmol L$^{-1}$, Dojindo B568-10, Munich, Germany) was dissolved in deionized H$_2$O, stored at ~80 °C and used after thawing. Na$_2$S (100 mmol L$^{-1}$) as a source of H$_2$S and polysulphides, sodium disulfide (Na$_2$S$_2$, 10 mmol L$^{-1}$) and sodium tetrasulfide (Na$_2$S$_4$, 10 mmol L$^{-1}$) (Dojindo SB01, SB02 and SB04, respectively, Munich, Germany) were prepared in argon-bubbled deionized H$_2$O, aliquoted, stored at ~80 °C and thawed just before use [5]. Na$_2$S dissociates in aqueous solution and reacts with H$^+$ to yield H$_2$S, HS$^-$ and a trace of S$^{2-}$. For simplicity, we use the terms H$_2$S to describe the total mixture of H$_2$S, HS$^-$ and S$^{2-}$ forms. Similarly, Na$_2$S$_2$ and Na$_2$S$_4$ dissociate in aqueous solution yielding S$_n$$^{2-}$, HS$^-$ and traces of H$_2$S$_n$ ($n = 2$ and 4). For simplicity, we use the terms Na$_2$S$_2$ and Na$_2$S$_4$. For EPR samples, buffer consisting of 50 mmol L$^{-1}$ sodium phosphate (pH 7.4, 37 °C) and 100 µmol L$^{-1}$ diethylenetriaminepentaacetic acid (DTPA) was used. Saturated KO$_2$/DMSO solution was prepared by the addition of powdered KO$_2$ (Sigma-Aldrich 278904, Steinheim, Germany) into the anhydrous DMSO (1.42 mg mL$^{-1}$, 23 ± 1 °C; theoretically 20 mmol L$^{-1}$ KO$_2$), vortexed for 2 min, sonicated for 20 s and let for 1 h to settle down the undissolved KO$_2$ powder. When an aliquot of KO$_2$/DMSO was taken from the bottom part of the stock solution and added into the phosphate buffer, the spectral intensity of •BMPO-OOH was 2- to 4-fold higher than that of the aliquot taken from the upper part [5,13]. For EPR study we used the aliquots of the saturated stock KO$_2$/DMSO obtained from the upper part of the stock solution. The intensity of •BMPO-OOH EPR spectra was reproducible when the KO$_2$/DMSO stock solution was incubated at 23 ± 1 °C and used within ~4 h.

2.2. EPR Study of the BMPO-Adducts

A modified protocol from our previous study was used [13]. First, •BMPO-OOH was prepared by the addition of the saturated KO$_2$/DMSO solution (10% v/v DMSO/final buffer) into the BMPO (30 mmol L$^{-1}$ final concentration, 37 °C) diluted in the phosphate buffer. The BMPO/KO$_2$ sample was mixed for 10 s and then the studied compounds, H$_2$Sn, SeO$_n^{2-}$ or H$_2$Sn/SeO$_n^{2-}$ mixtures, were added. The sample was mixed again for 5 s and transferred to a standard cavity aqueous EPR flat cell (WG 808-Q, Wilmad-LabGlass, Vineland, NJ, USA). The first EPR spectrum was recorded 100 s after the addition of the compounds. The sets of the individual EPR spectra of the BMPO spin-adducts were recorded as 15 sequential scans, each 42 s, with a total acquisition time of 11 min. Each experiment was repeated at least twice. EPR spectra of the BMPO spin-adducts were measured on a Bruker EMX spectrometer (Rheinstetten, Germany), X-band ~9.4 GHz, 335.15 mT central field, 8 mT scan range, 20 mW microwave power, 0.1 mT modulation amplitude, 42 s sweep time, 20.48 ms time constant and 20.48 ms conversion time at 37 °C. To compare the relative potency of the compounds to decrease overall trapped radical concentration, a second integral of the total EPR spectra intensity of the BMPO-adducts was evaluated. To obtain the •BMPO-OH/*BMPO-OOH radical proportion, the simulated spectra ratio was calculated using EasySpin program working on MatLab platform [22].
2.3. Plasmid DNA Cleavage

A pDNA cleavage assay with the use of pBR322 plasmid (New England BioLabs, Inc., N3033 L, Ipswich, MA, USA) was performed as reported previously [13]. In this assay, all samples contained 0.2 μg pDNA in the sodium phosphate buffer (25 mmol L\(^{-1}\) sodium phosphate, 50 μmol L\(^{-1}\) DTPA, pH 7.4). FeCl\(_2\) (150 μmol L\(^{-1}\)) was used in control experiments. Powdered KO\(_2\) was dissolved by 4 mmol L\(^{-1}\) BMPO in the buffer containing 10% DMSO (v/v), vortexed for 10 s and 10 μL of the mixture was added into 10 μL solution of pDNA. The resulting mixtures were incubated for 30 min at 37 °C. After incubation, the reaction mixtures were subjected to 0.6% agarose gel electrophoresis. Integrated densities of all pBR322 forms in each lane were quantified using the Image Studio analysis software (LI-COR Biotechnology, Bad Homburg, Germany) to estimate pDNA-cleavage efficiency.

3. Results

3.1. *BMPO-OOH as a Model Hydroperoxide and the Effects of Na\(_2\)S/Na\(_2\)SeO\(_3\)

KO\(_2\) in DMSO dissociates to K\(^+\) and relatively stable O\(_2^{•−}\), but its solubility in DMSO is extremely low [23,24]. EPR spectra after the addition of KO\(_2\) into the BMPO-buffered solution showed signals of two conformers of the *BMPO-OOH adduct, as a result of O\(_2^{•−}\) trapping by BMPO (Figure 1a1–a3). The *BMPO-OOH signal was relatively stable and slowly decreased (t\(_{1/2}\) ≈ 23 min) in accordance with [15,16,18]. However, when KO\(_2\) was added into the buffer first, followed by the addition of BMPO 10 s later no radical was trapped by BMPO, i.e., the EPR spectrum was not observed (Figure 1b1–b3). The results confirmed a short life time of O\(_2^{•−}\) in water solution (t\(_{1/2}\) ≈ 1–10 μs). Therefore, we used the interaction of O\(_2^{•−}\) with BMPO, producing the *BMPO-OOH spin-adduct, as a model of organic hydroperoxides to study the effects of Na\(_2\)S/Na\(_2\)SeO\(_3\). The following sequence of the compounds was used to prepare the sample: KO\(_2\) was added into BMPO-buffered solution, resulting in the formation of *BMPO-OOH and then the studied compounds were added 10 s later.

Information that can be obtained from the EPR spectra after the interaction of *BMPO-OOH with compounds added into the system is as follows: First, it is an effect of compounds on the total integral EPR intensity of the BMPO-adducts obtained as a second integral of EPR spectra. Second, from the simulation of individual BMPO-adducts of the experimental EPR spectra it may be possible to obtain the changes in their relative concentrations (*BMPO-OOH and *BMPO-OH), thereby reflecting the effect of compounds.

The presence of SeO\(_3^{2−}\) (25 μmol L\(^{-1}\)) did not influence the rate of *BMPO-OOH decomposition (Figure 1c1). From the similar shape of the first five cumulative spectra (Figure 1c2) and the last five accumulated spectra (Figure 1c3), it is assumed that there was no interaction between SeO\(_3^{2−}\) and *BMPO-OOH. The addition of Na\(_2\)S (25 μmol L\(^{-1}\); H\(_2\)S) slightly increased the rate of *BMPO-OOH decay (Figure 1d1), as an indication of scavenging/interaction of the BMPO-adducts by/with H\(_2\)S. Since the shape of the last five accumulated spectra (Figure 1d3) was different from the first five spectra (Figure 1d2), it can be concluded that H\(_2\)S affects the *BMPO-OOH spin-adduct. The details on the interaction are described in the next section. The H\(_2\)S/SeO\(_3^{2−}\) (25/25 μmol L\(^{-1}\)/μmol L\(^{-1}\)) mixture decreased *BMPO-OOH concentration during 100 s after the sample preparation (before EPR measurement started) and later the BMPO-adducts concentration was approximately constant (Figure 1e1). However, spectra were different (Figure 1e2,e3) from control *BMPO-OOH (Figure 1a2,a3), indicating interaction of H\(_2\)S/SeO\(_3^{2−}\) with *BMPO-OOH. Similar effect was detected when polysulfide Na\(_2\)S\(_2\) (25 μmol L\(^{-1}\)) and the Na\(_2\)S\(_2)/SeO\(_3^{2−}\) (25/25 μmol L\(^{-1}\)/μmol L\(^{-1}\)) mixture was used (Figure 1f1–f3,g1–g3). The effects of polysulfide Na\(_2\)S\(_2\) (25 μmol L\(^{-1}\)) was the most pronounced, it diminished *BMPO-OOH concentration during 100 s, before EPR measurement started (Figure 1h1–h3). However, its effect to scavenge the BMPO-adducts decreased when the Na\(_2\)S\(_2)/SeO\(_3^{2−}\) (25/25 μmol L\(^{-1}\)/μmol L\(^{-1}\)) mixture was used (Figure 1i1). The spectra (Figure 1i2,i3) revealed pronounced interaction with *BMPO-OOH.
From the simulation of individual BMPO-adducts of the experimental EPR spectra it may be possible to obtain the integral EPR intensity of the BMPO-adducts obtained as a second integral of EPR spectra. Second, the effects of compounds on the total intensity from the EPR spectra are as follows: First, it is an effect of compounds on the total intensity of the BMPO-adducts, each 42 s, with starting acquisition 100 s after sample preparation; (a1–i1) Collection of 15 EPR spectra arranged back-to-back of the BMPO-adducts, each 42 s, with starting acquisition 100 s after sample preparation; (a2–i2) first to fifth accumulated spectra; (a3–i3) last five accumulated spectra; (a1–a3) representative control EPR spectra from 2–3 measurements of •BMPO-OOH after saturated KO2/DMSO solution (final 10% v/v DMSO) was added to 30 mmol L−1 BMPO in the buffer consisting 50 mmol L−1 sodium phosphate buffer and 0.1 mmol L−1 DTPA (pH 7.4, 37 °C); spectra of *BMPO-OOH after the addition of (c1–c3) 25 µmol L−1 SeO32−, (d1–d3) 25 µmol L−1 Na2S, (e1–e3) 25/25 in (µmol L−1) Na2S2/SeO32−, (f1–f3) 25 µmol L−1 Na2S2, (g1–g3) 25/25 (in µmol L−1) Na2S2/SeO32−, (h1–h3) 25 µmol L−1 Na2S4 and (i1–i3) 25/25 (in µmol L−1) Na2S2/SeO32−. In the case of (b1–b3) spectra, 30 mmol L−1 BMPO was added 10 s after KO2/DMSO was mixed with the buffer. The intensities of the (a1–i1) time-dependent EPR spectra and (a2–i2, a3–i3) detailed spectra are comparable; they were measured under identical EPR spectrometer settings.

When SeO42− was used instead of SeO32−, it did not influence the effects of Na2S, Na2S2 and Na2S4, indicating no formation of redox active products of the Na2Sn/SeO42− interaction (Figure 2).

Figure 1. Electron paramagnetic resonance (EPR) spectra of •BMPO-OOH modulated by Na2S, Na2S2, Na2S4 and their interaction with SeO32−. (a1–i1) Collection of 15 EPR spectra arranged back-to-back of the BMPO-adducts, each 42 s, with starting acquisition 100 s after sample preparation; (a2–i2) first to fifth accumulated spectra; (a3–i3) last five accumulated spectra; (a1–a3) representative control EPR spectra from 2–3 measurements of •BMPO-OOH after saturated KO2/DMSO solution (final 10% v/v DMSO) was added to 30 mmol L−1 BMPO in the buffer consisting 50 mmol L−1 sodium phosphate buffer and 0.1 mmol L−1 DTPA (pH 7.4, 37 °C); spectra of •BMPO-OOH after the addition of (c1–c3) 25 µmol L−1 SeO32−, (d1–d3) 25 µmol L−1 Na2S, (e1–e3) 25/25 in (µmol L−1) Na2S2/SeO32−, (f1–f3) 25 µmol L−1 Na2S2, (g1–g3) 25/25 (in µmol L−1) Na2S2/SeO32−, (h1–h3) 25 µmol L−1 Na2S4 and (i1–i3) 25/25 (in µmol L−1) Na2S2/SeO32−. In the case of (b1–b3) spectra, 30 mmol L−1 BMPO was added 10 s after KO2/DMSO was mixed with the buffer. The intensities of the (a1–i1) time-dependent EPR spectra and (a2–i2, a3–i3) detailed spectra are comparable; they were measured under identical EPR spectrometer settings.
we analyzed all accumulated spectra by simulation. Figure 3 shows representative examples of the spectra (Table 1). BMPO-hydroxyl radical (BMPO-OOH) was the most pronounced, it

did not influence the rate of BMPO-OOH concentration during 100 s, before EPR measurement started (Figure 1d1). The spectra (Figure 1d2,d3) revealed pronounced

decay (Figure 1d1), as an indication of scavenging/interaction of the BMPO-adducts by/with H2S. Since the shape of the last five accumulated spectra (Figure 1d3) was different from the first five (Figure 1e1). However, spectra were different (Figure 1e2,e3) from control measurement started) and later the BMPO-adducts concentration was approximately constant (Figure 1f1–f3,g1–g3). The effects of polysulfide Na2S4 (25 µmol L−1) diminished indicating no formation of redox active products of the Na2Sn/SeO42− interaction with BMPO-OOH. The addition of Na2S (25 µmol L−1) resulted in the changes in their relative concentrations (•BMPO-OOH and •BMPO-OH were simulated considering presence of two conformers [15,18,19].

3.2. Simulation of BMPO-Adducts Spectra in the Presence of Na2Sn/Na2SeO3

The studied compounds changed shapes of EPR spectra of the BMPO-adducts, indicating the superposition of signals corresponding to the generation of individual BMPO-adducts. Therefore, we analyzed all accumulated spectra by simulation. Figure 3 shows representative examples of the simulation of the sixth to tenth accumulated spectra only. The results showed that the best fit was obtained when the hyperfine coupling constants for two conformers of •BMPO-OOH and two of BMPO-hydroxyl radical (•BMPO-OH) adducts, along with those of BMPO-adduct with carbon-centered radical (•BMPO-CR) were inserted in spin-Hamiltonian calculations. The simulated spectra shown in Figure 3 were calculated using the hyperfine coupling constants elucidated from the experimental spectra (Table 1).

Table 1. Hyperfine coupling constants of the BMPO spin-adducts elucidated from the simulations of experimental spectra measured in the buffer solutions containing KO2 and 10% DMSO (v/v).

| BMPO-Adduct     | aN, mT  | aHβ, mT  | aHy, mT  |
|-----------------|---------|---------|---------|
| •BMPO-OH(1)     | 1.423 ± 0.011 | 1.541 ± 0.014 | 0.078 ± 0.011 |
| •BMPO-OH(2)     | 1.365 ± 0.038 | 1.248 ± 0.036 | 0.073 ± 0.015 |
| •BMPO-OOH(1)    | 1.339 ± 0.002 | 1.186 ± 0.007 | –        |
| •BMPO-OOH(2)    | 1.334 ± 0.003 | 0.958 ± 0.007 | –        |
| •BMPO-CR        | 1.528    | 2.221    | –        |

Figure 2. EPR spectra of •BMPO-OOH modulated by the mixture of Na2S, Na2S2 and Na2S4 with SeO42−. Representative EPR spectra from 2–3 measurements of •BMPO-OOH obtained in KO2/DMSO solution (final 10% v/v DMSO) in the presence of 30 mmol L−1 BMPO prepared in buffer consisting 50 mmol L−1 sodium phosphate buffer and 0.1 mmol L−1 DTPA (pH 7.4, 37 °C), after the addition of (a1–a3) 25 µmol L−1 SeO42−, (b1–b3) 25/25 (in µmol L−1) Na2S/SeO42−, (c1–c3) 25/25 (in µmol L−1) Na2S2/SeO42− and (d1–d3) 25/25 (in µmol L−1) Na2S4/SeO42−. Sets of individual EPR spectra of the BMPO-adducts were recorded as described in legend to Figure 1. The intensities of the (a1–d1) time-dependent EPR spectra and (a2–d2, a3–d3) detailed spectra are comparable, they were measured under identical EPR spectrometer settings.
The studied compounds changed shapes of EPR spectra of the BMPO-adducts, indicating the studied compounds significantly.

For simplicity, the relative concentration of two conformers, *BMPO-OH(1) and *BMPO-OH(2), were summed up and described as *BMPO-OH. Analogously, *BMPO-OOH(1) and *BMPO-OOH(2) were summed up and described as *BMPO-OOH. The time dependence of such evaluated BMPO-adducts EPR integral intensity is shown in Figure 4. In this figure, the absolute integral of individual BMPO-adducts is depended besides sample composition also on time. For example, the first to fifth accumulated spectra of control integral intensity of *BMPO-OOH spectra (circle; measured 1.7–5.2 min after sample preparation; see Figure 4a tick 1–5) has value 100 (r.u.) and the integrals of *BMPO-OH (triangle) and *BMPO-CR (cross) components of the sample are close to zero. The same control sample is measured 5.2–8.7 min after sample preparation (sixth to tenth accumulated spectra; see Figure 4a tick 6–10) and shows decrease to 73.5 (r.u.) for *BMPO-OOH component, whereas integral of *BMPO-OH is close to zero and *BMPO-CR increased to 4.2.

In controls and samples with SeO$_3^{2-}$, similar concentration of radicals and a slow decay of the *BMPO-OOH component was seen, indicating no interaction of SeO$_3^{2-}$ with *BMPO-OOH (Figure 4a,b). The addition of Na$_2$S increased the rate of *BMPO-OOH decay (Figure 4c), whereas slightly increased the concentration of *BMPO-OH. The decay was several times pronounced when the H$_2$S/SeO$_3^{2-}$ mixture was used (Figure 4d). Na$_2$S$_2$ and Na$_2$S$_4$ alone and their mixture with SeO$_3^{2-}$ significantly

**Figure 3.** Representative normalized experimental EPR spectra of the BMPO-adducts along with their simulation using the hyperfine coupling constants summarized in Table 1. Experimental spectra of the sixth to tenth accumulated spectra are shown only (blue); simulated spectra are red. (a) Control 30 mmol L$^{-1}$ BMPO + KO$_2$ and after addition of (b) 25 µmol L$^{-1}$ SeO$_3^{2-}$, (c) 25 µmol L$^{-1}$ H$_2$S, (d) 25/25 (in µmol L$^{-1}$) H$_2$S/SeO$_3^{2-}$, (e) 25 µmol L$^{-1}$ Na$_2$S$_2$, (f) 25/25 (in µmol L$^{-1}$) Na$_2$S$_2$/SeO$_3^{2-}$, (g) 25 µmol L$^{-1}$ Na$_2$S$_4$ and (h) 25/25 (in µmol L$^{-1}$) Na$_2$S$_4$/SeO$_3^{2-}$.
decreased the number of radicals and increased the rate of *BMPO-OOH decay (Figure 4e–h), whereas slightly increased concentration of *BMPO-OH with exception of Na₂S₄, where radical concentration was close to zero (Figure 4g). It is noticed that spin adducts concentration in the case of Na₂S₄/SeO₃²⁻ was noticeably higher compared to Na₂S₄ alone (Figure 4g vs. Figure 4h). The total integral EPR intensity of all components (Figure 4) revealed that the order of potential to transform/scavenge the BMPO-adducts was Na₂S₄ > Na₂S₂ > H₂S (Figure 4c,e,g) and H₂S/SeO₃²⁻ > H₂S (Figure 4c,d), Na₂S₂ > Na₂S₂/SeO₃²⁻ (Figure 4e,f), but Na₂S₄ > Na₂S₄/SeO₃²⁻ (Figure 4g,h).

**Figure 4.** Comparison of integral EPR intensity of individual BMPO-adducts elucidated from the simulation of experimental EPR spectra. The first to fifth accumulated spectra (1–5; 1.7–5.2 min after sample preparation; see Figure 1), the sixth to tenth accumulated spectra (6–10; 5.2–8.7 min after sample preparation) and the eleventh to fifteen accumulated spectra (11–15; 8.7–12.2 min after sample preparation; see Figure 1) Spectral components: *BMPO-OOH* (blue), *BMPO-OH* (red) and *BMPO-CR* (green); n = 2–3. (a) Control 30 mmol L⁻¹ BMPO + KO₂ and after addition of (b) 25 µmol L⁻¹ SeO₃²⁻, (c) 25 µmol L⁻¹ H₂S, (d) 25/25 (in µmol L⁻¹) H₂S/SeO₃²⁻, (e) 25 µmol L⁻¹ Na₂S₂, (f) 25/25 (in µmol L⁻¹) Na₂S₂/SeO₃²⁻, (g) 25 µmol L⁻¹ Na₂S₄ and (h) 25/25 (in µmol L⁻¹) Na₂S₄/SeO₃²⁻. Data are presented as the means ± SEM.
In order to compare the relative ratio of components of the BMPO-adducts the sum of the radicals at each time was normalized to 100% (Figure 5). In controls and samples with SeO$_3^{2-}$, the major *BMPO-OOH component was relatively constant over the time with minor *BMPO-CR component, indicating no decomposition of *BMPO-OOH by SeO$_3^{2-}$ (Figure 5a,b). H$_2$S and the H$_2$S/SeO$_3^{2-}$ mixture decreased *BMPO-OOH component and increased *BMPO-OH over the time (Figure 5c,d), suggesting time-dependent transformation/decomposition of the model hydroperoxide *BMPO-OOH to *BMPO-OH. Similar qualitative results were observed when Na$_2$S$_2$ alone or in the combination with SeO$_3^{2-}$ was used (Figure 5e,f). Na$_2$S$_4$ alone significantly decreased *BMPO-OOH and increased the *BMPO-OH component (Figure 5g). The Na$_2$S$_4$/SeO$_3^{2-}$ mixture had similar, but slightly lower effects compared to Na$_2$S$_4$ (Figure 5h). The order of the compounds potency to cleave *BMPO-OOH, estimated from the time-dependent *BMPO-OH/*BMPO-OOH ratio was: Na$_2$S$_4$ > Na$_2$S$_4$/SeO$_3^{2-}$ > H$_2$S/SeO$_3^{2-}$ > Na$_2$S$_2$ ~Na$_2$S$_2$/SeO$_3^{2-}$ ~H$_2$S > SeO$_3^{2-}$ ~control.

Figure 5. Ratio of normalized integral EPR intensity of individual BMPO-adducts evaluated from experimental spectra simulation. Data calculated from Figure 4. For explanation of (a–h) and figure description, see legend to Figure 4.
3.3. Cleavage of Plasmid DNA

Since Na$_2$S interacted with $\cdot$BMPO-OOH (Figures 4 and 5), it was of interest to know whether radical species, which can damage DNA, were formed during the interaction. The procedure for EPR experiments was modified to observe pDNA cleavage. In the control experiment, Fe$^{2+}$ (150 µmol L$^{-1}$) cleaved pDNA. However, when the reaction buffer contained 10% (v/v) DMSO, Fe$^{2+}$ did not cleave pDNA. Cleaving of pDNA was observed neither in the presence of BMPO/KO$_2$ mixture in buffer with 10% (v/v) DMSO, nor when Na$_2$S was added into BMPO/KO$_2$/DMSO (Figure 6). From the experiments with Fe$^{2+}$ it is evident that DMSO interfered with the assay. This is supported also by information that DMSO is a scavenger of HO$^\cdot$ radicals [13,25]. Therefore, we did not precede with the pDNA experiments.

Figure 6. Effect of BMPO/KO$_2$ on pDNA cleavage in the absence and presence of Na$_2$S. Representative gel (a) and column graph (b) indicating control (black) and the effects of Fe$^{2+}$ (150 µmol L$^{-1}$ FeCl$_2$) in 25 mmol L$^{-1}$ sodium phosphate buffer and 50 µmol L$^{-1}$ DTPA (pH 7.4) without (dark) and with 10% (v/v) DMSO (gray) and increasing concentrations of Na$_2$S on pDNA cleavage in the phosphate buffer containing 10% DMSO (v/v). The band at the bottom corresponds to the circular supercoiled form of pDNA and the less intense band appearing above in the case of Fe$^{2+}$ (dark column) represents the linear form of pDNA. The top band corresponds to the nicked circular form of pDNA. Values are the means ± SEM, n = 3.
4. Discussion

In our previous study, when KO$_2$ as a source of O$_2$•$^-$ was added into the mixture of H$_2$S/BMPO or H$_2$S/SeO$_3^{2-}$/BMPO, the concentration of trapped radicals changed and a superposition of several individual BMPO-adducts was detected [13]. It was not clear which components of the spectra resulted from the compounds/O$_2$•$^-$ or from the compounds/BMPO-OOH interaction. Therefore, in the present work we used a different approach, which allowed us to study interaction of the compounds with a model of organic hydroperoxide •BMPO-OOH. This approach is based on the short lifetime of O$_2$•$^-$ in water solution and relatively stable •BMPO-OOH to which the studied compounds were added.

This approach allowed the study of the effects of compounds on decomposition/transformation of the organic hydroperoxide •BMPO-OOH to the ratio of •BMPO-OH/•BMPO-OOH, which was detected by EPR. To elaborate this, we performed a detailed simulation of the experimental EPR spectra obtaining the hyperfine coupling constants of the individual BMPO-adducts under these experimental conditions, along with their relative concentrations. From the comparison of the experimental and the simulated EPR spectra (Figure 3), it can be concluded that the BMPO-adducts spectra were very well simulated by the hyperfine coupling constants (Table 1), based on the presence of two conformers, •BMPO-OH(1) and •BMPO-OH(2), •BMPO-OOH(1) and •BMPO-OOH(2) and •BMPO-CR. The hyperfine coupling constants were clearly indicated from the spectra simulations (Table 1) and were comparable to the published BMPO-adducts. The relative intensity of •BMPO-OOH decreased slowly over time and was comparable, but not identical, to the reported values under physiological conditions without DMSO [15,16,18]. It is likely that the •BMPO-CR component resulted from the trapping carbon-centered radical, originating from DMSO. From the time dependence of the integral EPR intensity (Figures 4 and 5) it was possible to evaluate the time-dependent effects of the compounds on the total BMPO-adduct concentration and the •BMPO-OH/•BMPO-OOH ratio, which is suggested to be proportional to the organic hydroperoxide •BMPO-OOH transformation/decomposition. The presence of SeO$_3^{2-}$ potentiated the decrease of integral intensity of spin-adducts induced by Na$_2$S, had no significant effect on Na$_2$S$_2$ potency, but decreased the potency of Na$_2$S$_4$ (Figure 4). These results indicate that the interaction of SeO$_3^{2-}$ with Na$_2$S$_n$, which leads to the formation of redox radical species significantly depends on the number of S atoms.

The order of ability to decrease the total integral intensity of the BMPO-adducts (Figure 4) of H$_2$S/SeO$_3^{2-}$ > H$_2$S or Na$_2$S$_4$ > H$_2$S was similar to the order when KO$_2$ was added to the mixtures of the BMPO/compounds or DEPMPO/compounds [5,13]. This indicates that in the case when KO$_2$ was added to the mixtures of spin trap/compounds, the compounds affected mostly •BMPO-OOH or •DEPMPO-OH radicals.

A comparison of the time-dependent ratio of •BMPO-OH/•BMPO-OOH (Figure 5) revealed that the increase of the •BMPO-OH spectral component was at the expense of the •BMPO-OOH component, supporting the concept of decomposition/transformation of organic hydroperoxide. Interactions of H$_2$S and polysulfides with radical species are complex [4,7] and more studies are needed to explain the most profound potential of Na$_2$S$_4$ > Na$_2$S$_2$ or effects of SeO$_3^{2-}$ to decrease the potency of Na$_2$S$_4$ to cleave •BMPO-OOH.

In conclusion, •BMPO-OOH was demonstrated to be a helpful model of organic hydroperoxide. The presented approach of EPR spectra measurement and analysis of the time-dependent ratio of the •BMPO-OH/•BMPO-OOH spin-adducts utilizing the spectra simulation could be useful to study potential of compounds to transform/decompose •BMPO-OOH. Using this approach, the impact of sulfide derivatives (Na$_2$S$_n$) alone or in the combination with SeO$_3^{2-}$ to transform/decompose •BMPO-OOH was detected and compared. Since 10% DMSO (v/v) is used in the studied system, the procedure is useful for evaluating antioxidant potency of compounds insoluble in water.

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