

Catalytic Generation of Superoxide by Different Alcohols

Anuradha Anand Shastri, Julian Ernest Spallholz*

ABSTRACT

Objective: Group VIA elements, oxygen, sulfur and selenium can be toxic and many of their compounds are toxic to cells owing to the catalytic generation of superoxide and oxidative stress from thiol oxidations. Sulfides, (RS-) and selenides, (RSe-) of organic molecules and enzymes are often redox catalysts. In the current study, alcohols (ROH) were investigated to ascertain if oxides (RO-) of some alcohols might also ionize and redox cycle generating superoxide. **Methods:** The Lucigenin chemiluminescence assay was used for the detection of superoxide generation by the aliphatic alcohols and Benzyl Alcohol at 25°C and 37°C in the presence or absence of reduced glutathione (GSH). Similar Benzyl compounds of sulfur, selenium and oxygen were also tested for direct comparison of their catalytic activity. **Results:** Many of the alcohols tested, generated superoxide in the presence of GSH at both 25°C and 37°C, but not in the absence of GSH. Overall catalytic activity was greater at 37°C than at 25°C. Comparing the catalytic activity of equal concentrations of the S, Se and O moiety of the Benzyl compounds showed that although the catalytic alcohols did generate superoxide in the presence of GSH, but the sulfur and selenium compounds showed greater catalytic activity. **Conclusion:** As hypothesized, some aliphatic alcohols tested did generate superoxide similar to many sulfur and selenium analog compounds in the presence of GSH. From the results we can deduce that some alcohols may be following a redox mechanism that is similar to the S and Se compounds that redox cycle in presence of GSH generating superoxide.

Key words: Alcohols, Superoxide, Redox Cycling, Chemiluminescence, Reduced Glutathione (GSH), Sulfur, Selenium.

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INTRODUCTION

The Chalcogens, Group VIA of the Periodic Table contains 5 elements, oxygen (O), sulfur (S), selenium (Se), tellurium (Te) and polonium (Po). The first three, O, S and Se are all biologically essential elements, minimally components of amino acids and proteins. Te is not known to be biologically required and Po is radioactive. In the seemingly simplest chemical ionized forms, sulfur (S) and selenium (Se), as sulfides and selenides are toxic and generate superoxide, (O₂⁻) and other ROS in the presence of reduced Glutathione (GSH) and other thiols.^{1,2}

Selenium (Se) and its compounds since the 1930's have long been known to be toxic to plants and animals.³ The more toxic Se compounds include inorganic selenite, methylselenide and the diselenides; selenocystamine and diselenodipropionic acid among others and all have been shown to generate superoxide by oxidation of reduced Glutathione (GSH) and other thiol oxidations.^{1,2,4} Selenium's cytotoxicity, like various sulfide toxicities, can be attributed to its lower catalytic selenide ionization, H₂Se, HSe-, pKa, 3.8. Ionized allyl sulfides and allyl selenides have been shown to be the cause for apoptosis in cancer cells.^{5,6} Isothiocyanates and isoselenocyanates as shown by Crampsie *et al.* are likewise catalytic and toxic generating O₂⁻.^{7,8} These

catalytic selenium selenides, perselenides, (GSSe-), isothiocyanates, sulforaphane and isoselenocyanates all oxidize GSH and other thiols producing O₂⁻ and other oxygen free radicals, accounting for their cytotoxicity and induction of apoptosis in cancer cells.⁹⁻¹³

Aliphatic alcohols and phenolic alcohols are organic compounds having a hydroxyl functional group (ROH), comparable to catalytic sulfides (RS-) and selenides (RSe-). Noting the structural similarity and known toxicity of alcohols we sought to survey several alcohols, ROH, to see if they like sulfides and selenides were capable of redox cycling upon ionization which had been reported for ethanol as far back as 1922.¹⁴ The possibility existed therefore, that alcohols may disassociate in neutral pH buffers to form catalytic alkyl oxides, RO- and redox cycle generating O₂⁻ and other ROS. In this survey of alcohols, we examined selected water soluble aliphatic and aromatic alcohols for their ability to redox cycle in the presence of GSH and generate detectable amounts of superoxide using chemiluminescent luminometry.

MATERIALS AND METHODS

All aliphatic, aromatic alcohols and other compounds, Lucigenin (Bis-N-Methylacridinium Nitrate), reduced

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glutathione (GSH), were reagent grade purchased from Sigma Chemical Co. (St. Louis, MO) except Pentanol that was a gift from Dr. Guigen Li of the Department of Chemistry, Texas Tech University.

Detection of Superoxide *in vitro*: Lucigenin Chemiluminescence (CL)

A cocktail containing Lucigenin (Bis-N-Methylacridinium Nitrate) 20 µg/mL and GSH, 1 mg/mL in 0.05 M phosphate buffer (pH 7.4) was used for the detection of superoxide ($O_2^{\cdot-}$) in the CL assay. The CL assay was conducted using a Turner Biosystems Luminometer Model TD-20e attached to an LKB Multitemp Model 2209 circulating water bath with thermometer. CL was recorded in 12 × 75 mm polystyrene tubes (Evergreen Scientific Co., Los Angeles, CA) at 25°C and 37°C over 30 sec integration periods with a 3 sec delay between integrations as previously performed for selenides.¹⁵ Chemiluminescence of the alcohols at 25°C and 37°C was recorded every 30 sec for 4-5 min. Chemiluminescence of alcohols alone at 25°C was done by adding 200 µL of the alcohols; Methanol, Ethanol, Propanol, Butanol, Pentanol, Hexanol, Heptanol, Octanol and Benzyl Alcohol to the polystyrene tubes and recording CL readings for 4-5 min. Chemiluminescence of alcohols alone at 37°C was done in a comparable manner with 200 µL of the alcohols. To measure $O_2^{\cdot-}$ CL generation, either 35 or 50 µL of the alcohols were added to the complete Lucigenin CL cocktail with reduced glutathione (GSH). The Lucigenin cocktail alone with or without reduced glutathione was used as the CL controls. Sodium selenite was added as the positive control for superoxide generation from the complete cocktail, as selenite rapidly forms GSSe-, with redox cycling generating much superoxide (4). To assess if $O_2^{\cdot-}$ was generated from and the source of CL from the alcohols, 25 or 50 µL of Heptanol which generated superoxide was added to the CL cocktail with GSH and CL was tested. To the Heptanol 2 mg of ascorbic acid or 1 mg of superoxide dismutase (SOD) was added to the cuvette and mixed during the chemiluminescence generation. If $O_2^{\cdot-}$ is present, CL is quenched by either ascorbic acid or SOD during selenite or other selenium compound generated CL.

The compounds, Benzyl Thiocyanate, Benzyl Selenocyanate and Benzyl Alcohol, each with sulfide, selenide or putative oxide anion were compared by CL to determine their ability to generate superoxide. Equivalent stock solutions of 2 mg/mL of RS-, RSe- and RO- compounds were prepared and CL assays.

Statistical analysis

All statistical analyses were done with the SPSS 23 statistical package. One-way ANOVA was performed to compare the means of the different aliphatic alcohols and Benzyl Alcohol at 25°C and 37°C, Tukey's HSD was used as the post hoc test. Means \pm SE were calculated and mean CL differences were considered significant if $p \leq 0.05$.

RESULTS

All aliphatic and aromatic alcohols tested alone (200 µL) showed no CL, i.e., no $O_2^{\cdot-}$ generation at 25°C. At 37°C some measurable chemiluminescence was seen for Butanol and Hexanol and this could be attributed to possible self-ionization at the higher temperature and loss of an electron to Lucigenin not seen at 25°C or with the other alcohols (Table 1).

When assessed for CL generation in the presence of GSH, 50 µL of alcohol was added to the CL cocktail resulting in the generation of CL data, indicative of some alcohols generating superoxide at 25°C. Three even carbon numbered alcohols, Ethanol, Butanol and Hexanol were chemiluminescent whereas the water soluble odd carbon numbered alcohols; Methanol, Propanol and Pentanol were not CL with the exception of Heptanol which generated chemiluminescence (Figure 1). Pentanol and Hexanol generated only very slight CL as the solubility in aqueous buffer

Table 1: Chemiluminescence of 200 µL of the C1-C8 alcohols at 25°C and the C1-C6 alcohols at 37°C alone compared to CL blank. All values are relative CLUs $\times 10^3$, N=10.

Alcohol	Temperature	
	25°C	37°C
Blank	0.04	0.06
Methanol	0.04	0.05
Ethanol	0.04	0.12
Propanol	0.05	1.19
Butanol	0.05	12.02
Pentanol	0.02	0.45
Hexanol	0.04	6.86
Heptanol	0.02	-
Octanol	0.04	-

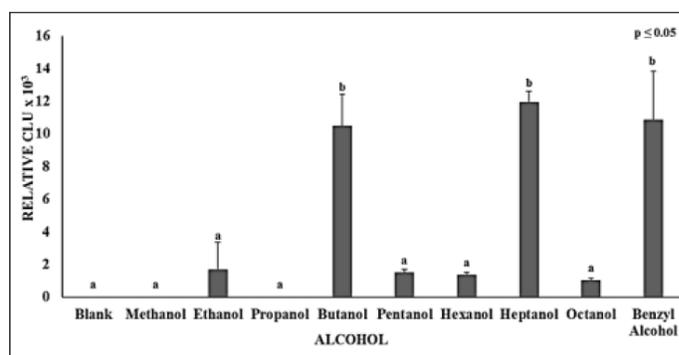


Figure 1: CL of 50 µL of the C1-C8 aliphatic alcohols and Benzyl Alcohol added to the CL cocktail (500 µL) at 25°C. CL was recorded as described above. Different letters designate significant differences in chemiluminescent activity among the aliphatic alcohols and Benzyl Alcohol. The same letters, a or b, designate no significant differences in the Mean \pm SE of chemiluminescent activity, $p \leq 0.05$, N=10.

of these larger aliphatic alcohols is likely reduced (Figure 1). Similar, but not identical CL activity results were observed for the alcohols at 37°C (Figure 2). Temperature modified the CL activity of the alcohols with 4-carbon Butanol becoming the most CL active aliphatic alcohol tested at both temperatures. Benzyl Alcohol was not catalytic alone or in the absence of GSH, but was highly catalytic at temperatures, 25 and 37°C (Figures 1 and 2) in the presence of GSH. Phenol (data not shown) was not catalytic and produced no CL under any conditions or temperatures tested.

For one alcohol showing modest CL activity in the qualitative CL assay, Hexanol CL from $O_2^{\cdot-}$ generation was shown to be concentration dependent with a correlation coefficient of, R=0.95 (Figure 3).

For a direct comparison of the catalytic activity of available and similar S, Se and OH compounds, Benzyl Thiocyanate, Benzyl Selenocyanate and Benzyl Alcohol were compared for superoxide CL generation. As anticipated, the concentrations tested for CL were higher for the S and Se compounds than Benzyl Alcohol (Table 3).

Selenium and sulfur redox cycling compounds generating $O_2^{\cdot-}$ are quenched by superoxide dismutase (SOD) and ascorbic acid in a concentration dependent manner. Heptanol CL, one of the better redox cycling alcohols, had its superoxide generation quenched by ascorbic acid (Figure 4) but not SOD (Figures 5 and 6).

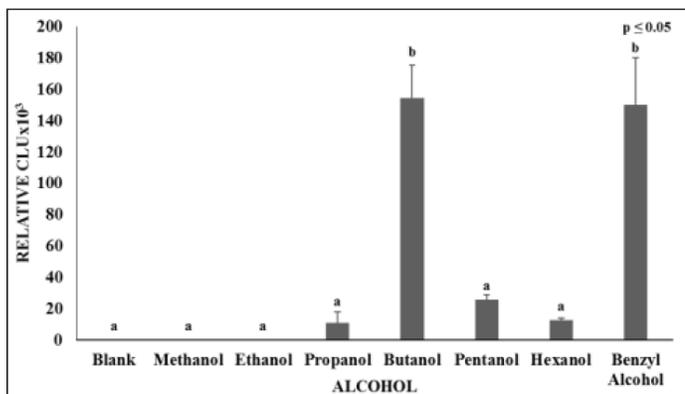


Figure 2: CL of 50 μ L of the C1-C6 aliphatic alcohols and Benzyl Alcohol were added to the CL cocktail (500 μ L) at 37°C. CL was recorded as described above. Different letters designate significant differences in chemiluminescent activity among the aliphatic alcohols and Benzyl Alcohol. The same letters, a or b, designate no significant differences in the Mean = +/- SE of chemiluminescent activity, $p \leq 0.05$, $N=20$.

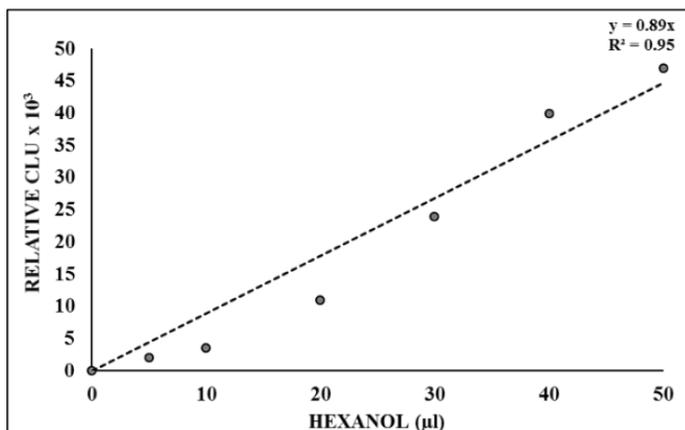


Figure 3: Correlation Coefficient ($R = 0.95$) of Hexanol Chemiluminescence at 37°C CL cocktail vs. summed CLUs over 10 min, $N=20$.

Table 2 shows that for all alcohols except Hexanol, chemiluminescent activity was absent or very low in the absence of GSH in the cocktail at 25°C and 37°C. Again, this anomaly of just Hexanol CL in the absence of GSH could be attributed to possible greater self-ionization and loss of an electron not seen with the other alcohols.

The overall chemiluminescent activity of the alcohols was much greater at 37°C (Figure 2) than at 25°C (Figure 1). At 37°C when alcohols were added to the cocktail containing GSH and Lucigenin, maximum chemiluminescent activity was seen for Butanol and Benzyl Alcohol. At 25°C a large spike in chemiluminescent activity was seen with Ethanol but it lasted only for the first 30 sec or less of the total assay time. At 25°C, maximum comparative chemiluminescent activity was seen for Butanol, Heptanol and Benzyl Alcohol.

Table 3 shows that at 25°C Benzyl Selenocyanate showed most chemiluminescent activity at all concentrations, while at 37°C Benzyl Thiocyanate showed the most chemiluminescent activity at all concentrations.

Figure 4 shows that the addition of ascorbic acid to the CL cocktail with Heptanol causing a sharp decrease in chemiluminescent activity, indicative of superoxide quenching.

Table 2: Chemiluminescence of C1-C6 alcohols at 25°C and 37°C in the absence of reduced glutathione (GSH). At 25°C, 35 μ L of each alcohol was added to the CL cocktail without GSH and at 37°C, 50 μ L was added to the CL cocktail without GSH. All values are relative CLUs x 10³, $N=10$.

Alcohol	Temperature	
	25°C	37°C
Blank	0.05	0.07
Methanol	0.04	1.71
Ethanol	0.04	1.2
Propanol	0.04	1.25
Butanol	0.21	1.15
Pentanol	0.53	4.9
Hexanol	25.6	33.68

Table 3: Relative Chemiluminescence of Benzyl Thiocyanate, Benzyl Selenocyanate and Benzyl Alcohol at equivalent concentrations of the S, Se and OH moiety respectively, at 25°C and 37°C with complete CL cocktail. All values are the mean CLU x 10³, $N=10$.

	25°C			37°C		
	Blank (Methanol) Chemiluminescence (CLUx 10 ³)	0.02	0.03	0.00	0.064	0.142
Benzyl Thiocyanate (S moiety)	43 μ g	86 μ g	129 μ g	62.5 μ g	125 μ g	500 μ g
Chemiluminescence (CLUx 10 ³)	0.05	0.18	0.20	0.11	0.33	4.31
Benzyl Selenocyanate (Se moiety)	80 μ g	160 μ g	240 μ g	62.5 μ g	125 μ g	500 μ g
Chemiluminescence (CLUx 10 ³)	0.03	0.16	2.00	0.07	0.07	0.32
Benzyl Alcohol (OH moiety)	30 μ g	60 μ g	90 μ g	62.5 μ g	125 μ g	500 μ g
Chemiluminescence (CLUx 10 ³)	0.08	0.02	0.05	0.07	0.07	0.15

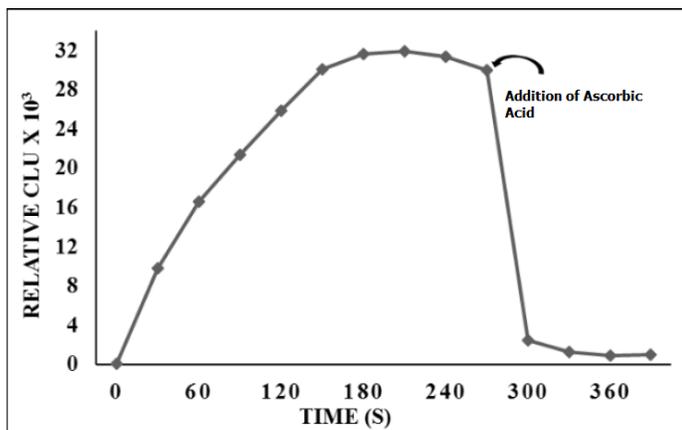


Figure 4: Mean CL of 50 μL of Heptanol added to the CL cocktail (500 μL) at 25°C. CL was recorded as described above and 2 mg ascorbic acid was added to cocktail at 270 secs, $N=16$.

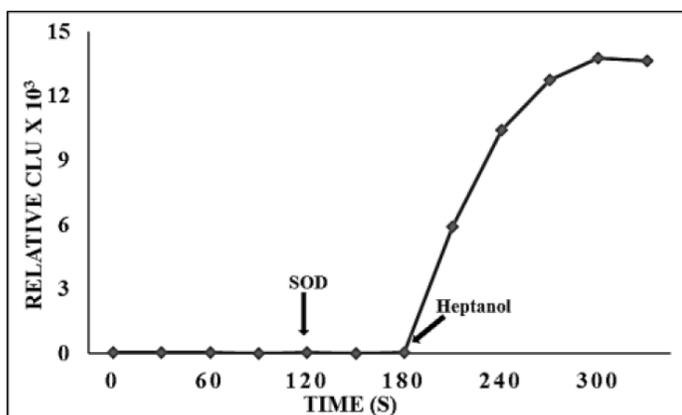


Figure 5: CL of 25 μL of Heptanol added to the CL cocktail (500 μL) at 25°C at 180 secs, following the addition of SOD to the cocktail at 120 secs, $N=16$.

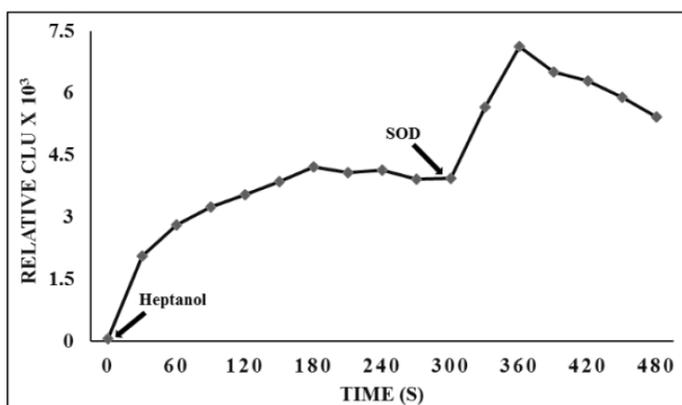


Figure 6: CL of 25 μL of Heptanol added to the CL cocktail (500 μL) at 25°C at 0 secs followed by addition of SOD to the cocktail at 300 secs, $N=16$.

Figure 5 and Figure 6 show an increase in chemiluminescence activity when superoxide dismutase is added before or after addition of Heptanol to the CL cocktail. Denaturation of SOD by the Heptanol is suspected in not preventing but increasing the $\text{O}_2^{\cdot-}$ generation in the CL quenching experiments.

DISCUSSION

Among the Group VIA compounds, cysteine and selenocysteine are amino acids participating in enzymatic redox cycling reactions. This redox catalytic property is the reason these amino acids are active at the sites of many enzymes; i.e., Urease and the Glutathione peroxidases.^{16,17} Other small organic S and Se compounds of the oxidation states, RS^{\cdot} and RSe^{\cdot} , often account for the generation of $\text{O}_2^{\cdot-}$ from the oxidation of GSH and other cellular thiols acting as "oxidases".^{18,19} The degree to which S, Se compounds and some alcohols are observed here to redox cycle and generate superoxide, in this Lucigenin CL *in vitro* system (Figure 3), leads one to assume that toxicity from at least some alcohols may also be a partial consequence of oxidative stress from alcohols in cells from $\text{O}_2^{\cdot-}$, superoxide generation. Redox cycling generating $\text{O}_2^{\cdot-}$ is dependent on molecular organic structure, making acute and chronic exposure of sulfides, isothiocyanates, selenides, isoselenocyanates or alcohols, cytotoxic.

The redox CL data for alcohols is not strikingly different from sulfide and selenide compounds, that have a structural catalytic similarity. For the alcohols the even numbered aliphatic alcohols, 2, 4 and 6 carbon alcohol generally exhibit more catalytic activity as measured by CL than the odd numbered carbon alcohols. A very recent study has reported that n-Butanol, a C4 alcohol, generating $\text{O}_2^{\cdot-}$ in the present experiments, has anti-tumor activity in mice.²⁰ Numerous sulfide and selenide compounds have been shown to be cytotoxic to cancer cells in culture and reduce cell growth in animals.^{8,21} Using Hexanol (C6 alcohol) CL was shown to be linear with concentration; $R=0.95$, as are selenium compounds. Within the aromatic compounds, Phenol was not catalytic under any conditions measured, whereas Benzyl alcohol was catalytic but not as much as Benzyl sulfide or Benzyl selenide.

Quenching of chemiluminescent activity for S and Se redox compounds has been observed using superoxide dismutase (SOD) or ascorbic acid. With the redox cycling alcohols nearly zero CL was observed when 2 mg of ascorbic acid was added to the CL cocktail containing Heptanol. This observation is suggestive that Heptanol, is indeed generating $\text{O}_2^{\cdot-}$ as the quenching of chemiluminescence was nearly 100% (Figure 4). However, superoxide dismutase, unlike with aqueous sulfur and selenium redox compounds, was unable to quench the $\text{O}_2^{\cdot-}$ being generated by Heptanol. In fact, there was no $\text{O}_2^{\cdot-}$ quenching of CL by Heptanol when SOD was added to the cocktail prior to the addition of Heptanol. When SOD was added during the CL assay of Heptanol there was an increase in CL activity. (Figure 5 and Figure 6). This anomaly suggests that Heptanol is likely denaturing the catalytic activity of the SOD, the SOD having no effect on CL when present before or added after the addition of Heptanol likely causing protein denaturation releasing Fe^{2+} and/or Cu^+ ions, which amplified the CL activity. In purely aqueous buffer systems SOD quenches $\text{O}_2^{\cdot-}$ and the CL activity of both S and Se redox cycling compounds.⁴

In final consideration of the CL activity of alcohols, noteworthy are the catalytic studies of S and Se analogs by Sharma *et al.*⁸ and by the animal Se toxicity studies of Schwarz *et al.*²² In both circumstances, there are clear structural catalytic/toxicity functional relationships within a series of similar S and Se compounds differing only in the R substitution. Similar functional relationships appear here with alcohols as generally shown for the catalytic and non-catalytic alcohols.

CONCLUSION

In conclusion, the experimental results presented suggest that there are differences in the ability of water soluble aliphatic and aromatic alcohols to partially ionize or in other ways oxidize GSH at physiological pH by forming the oxyl anion, $\text{RO}^{\cdot-}$, redox cycle and generate $\text{O}_2^{\cdot-}$ in concert

with its Group VIA sulfide and selenide compounds. Catalysis by thiols and selenides, generating $O_2^{\cdot-}$ and other ROS may help in explaining the toxicity of alcohols, i.e. aspects of a common association of antibacterial activity, general cellular and liver toxicity, from compounds of these three Group VIA elements.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

GSH: Reduced Glutathione; **S:** Sulfur; **Se:** Selenium; **RS:** Alkyl Sulfide; **RSe:** Alkyl Selenide; **RO:** Alkyl Oxide; **ROH:** Alcohol; **SOD:** Superoxide Dismutase; **CL:** Chemiluminescence; **ANOVA:** Analysis of Variance.

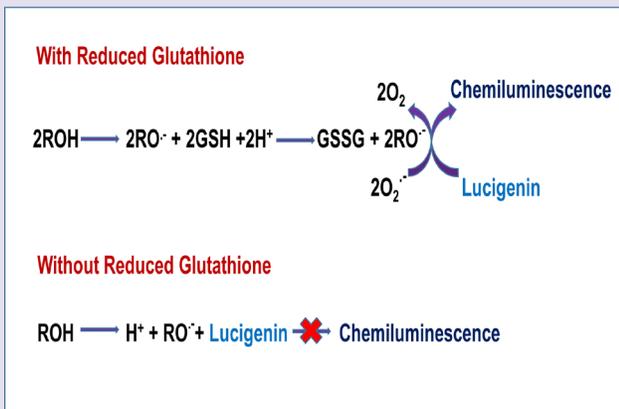
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GRAPHICAL ABSTARCT



SUMMARY

- Some compounds of Group VIA elements S and Se redox cycle in presence of GSH generating superoxide.
- Alcohols, compounds containing Oxygen, another element of Group VIA, were tested in the present study to determine if they too generate superoxide similar to catalytic S and Se compounds.
- From the results it can be concluded that some aliphatic and aromatic alcohols do generate superoxide in the presence of GSH.
- Amount of superoxide generated by alcohols is temperature dependent, more superoxide was generated at 37°C than 25°C.

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Julian E. Spallholz, did his graduate work in Biochemistry-Biophysics at Colorado State University in 1968; and Ph.D in University of Hawaii in 1971. He worked with the late Klaus Schwarz (1974-1978) that discovered the biological requirement of selenium and became a faculty member at Texas Tech, 1978 where he has spent 40 years teaching and researching the nutritional and toxicological properties of selenium. He is the author or co-author of ~ 80 publications, 200 abstracts, 15 US and Foreign Patents, books and scientific Proceedings. His retirement from Texas Tech University is expected in 2019.

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