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Metal-Sulfide Mineral Ores, Fenton Chemistry and Disease – Particle Induced Inflammatory Stress Response in Lung Cells

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Abstract

The inhalation of mineral particulates and other earth materials, such as coal, can initiate or enhance disease in humans. Workers in occupations with high particulate exposure, such as mining, are particularly at risk. The ability of a material to generate an inflammatory stress response (ISR), a measure of particle toxicity, is a useful tool in evaluating said exposure risk. ISR is defined as the upregulation of cellular reactive oxygen species (ROS) normalized to cell viability. This study compares the ISR of A549 human lung epithelial cells after exposure to well-characterized common metal-sulfide ore mineral separates. The evaluation of the deleterious nature of ore minerals is based on a range of particle loadings (serial dilutions of 0.002 m²/mL stock) and exposure periods (beginning at 30 minutes and measured systematically for up to 24 hours). There is a wide range in ISR values generated by the ore minerals. The ISR values produced by the sphalerite samples are within the range of inert materials. Arsenopyrite generated a small ISR that was largely driven by cell death. Galena showed a similar, but more pronounced

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Authors' contributions

ADH helped design the study, performed the experiments, and drafted the manuscript. AS performed experiments and edited the manuscript. SET and MAAS helped design the experiments, supervised the study, and edited the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

response. Copper-bearing ore minerals generated the greatest ISR, both by upregulating cellular ROS and generating substantial and sustained cell death. Chalcopyrite and bornite, both containing ferrous iron, generated the greatest ISR overall. Particles containing Fenton metals as major constituents produce the highest ISR, while other heavy metals mainly generate cell death. This study highlights the importance of evaluating the chemistry, oxidation states and structure of a material when assessing risk management.

Keywords

Ore minerals; Fenton Chemistry; Oxidative Stress; Inflammatory Stress Response; Occupational Disease

1. Introduction

From flint in the Neolithic period, gold and silver in the Iron Age, lead during Antiquity, to Rare Earth minerals in the present, mining of mineral resources has enabled important technological advances, fostered trade, as well as provided the raw materials for the creation of art and artifacts. Presently, mining of metals and mineral ores is a multibillion dollar industry, with metals and aggregates/industrial minerals generating a \$230 billion annual profit (MJO, 2012). While a crucial pillar of our society, mining of mineral resources has significant environmental and health issues associated with it. Exposure to mine dust has long been recognized as an occupational health hazard. Historically, exposure to quartz, asbestos particles and coal dust has received considerable attention given the prevalence of silicosis, asbestosis and coal workers' pneumoconiosis worldwide. However, far less is known about the inhalation risks associated with other earth materials that are mined and processed. While there have been some studies about the role of Fenton chemistry in the development or enhancement of disease (Winterbourn, 1995), there is much to be explored. The purpose of this study is to investigate the toxicity of common metal-sulfide minerals (copper-sulfides, copper-iron-sulfides, arsenic-iron-sulfide, lead-sulfide, and zinc-sulfides) that are mined or present in ore deposits as gangue minerals. Each of the materials evaluated is briefly described below.

1.1. Selected Metal-sulfides

Sphalerite (ZnS) is the main zinc ore mineral and commonly contains 26 mole % iron in its crystal structure ($\text{Zn}_{1-x}\text{Fe}_x\text{S}$) (Labrenz et al., 2000; Wright and Gale, 2010), with reports of up to 56 mole % (Di Benedetto et al., 2005; Patrick et al., 1998). Often associated with sphalerite, galena (PbS) is the main lead ore mineral. The cations for both ores are present in the divalent oxidation state. While arsenic is no longer mined, its main ore mineral, arsenopyrite (FeAsS), is often found in gold ores. As a result, a considerable amount of arsenopyrite is processed in the mining of gold. The oxidation state of iron in arsenopyrite is predominantly divalent (i.e., $\text{Fe}^{2+}(\text{AsS})^{-2}$) with a minor contribution of trivalent iron (i.e., $\text{Fe}^{3+}(\text{AsS})^{3-}$). Although the presence of either an arsenic and sulfur dianionic or trianionic group in the crystal structure is debated, when dispersed in solution, arsenopyrite releases ferrous iron (Asta et al., 2010). This suggests that iron in the arsenopyrite crystal structure is mostly in the divalent oxidation state. The oxidation states of copper and iron in chalcopyrite

(CuFeS₂), the primary copper ore mineral, has been extensively studied. Most studies indicate that the copper present is monovalent, although in oxidized surface layers divalent copper may be prevalent. To balance charge, trivalent iron is formally present in chalcopyrite, but charge transfer between iron and sulfur gives iron a strong divalent character (Goh et al., 2006; Todd et al., 2003). Furthermore, as with arsenopyrite, chalcopyrite releases ferrous iron into solution, which is important in the context of this study as the release of ferrous iron will allow the Fenton reaction to go forward:

Pyrite (FeS₂), as the most common sulfide mineral on Earth (and a potent reactive oxygen species generator (Cohn et al., 2006; Schoonen et al., 2006; Schoonen et al., 2010)), is often associated in varying abundances with all the ores described above.

Chalcocite (Cu₂S) and bornite (Cu₅FeS₄) are also important copper ores and are common weathering products of chalcopyrite. While copper in chalcocite is monovalent, it is possible that bornite contains a small amount of divalent copper as well. Iron in bornite has a formal trivalent oxidation state (Goh et al., 2006).

1.2. Particle Toxicity

The toxicity of particles can be derived by particle overload or from the reactivity of the material (Fubini, 1997; Morrow, 1988; Oberdorster, 1995). When particle overload occurs, the alveolar macrophage (AM)-mediated clearance is impaired, which leads to an accumulation of particles within the lungs. An excessive particle burden can cause an array of pulmonary issues, ranging from chronic inflammation and fibrosis, to the development of tumors (Oberdorster, 1995). Once inhaled, a mineral particle might undergo surface-mediated reactions, including dissolution reactions (Fubini, 1997; Ghiazza et al., 2011; Harrington et al., 2012a; Schoonen et al., 2006). The reactivity of an inhaled particle is a function of its stability in the lung environment, its solubility, and modifications of its surface, such as the presence of defects or surface coatings (Fubini, 1997; Ghiazza et al., 2011; Schoonen et al., 2006). The incomplete reduction of oxygen on the surface of the particles or by metals released from the particles will generate reactive oxygen species (ROS) (Schoonen et al., 2006). Furthermore, although tightly regulated, cells also generate ROS when under stress, possibly contributing to the oxidative stress (Araujo and Nel, 2009; Gloyne et al., 2011; Knight, 2000). Apart from oxidative damage, reactive materials also impair AM-mediated clearance at a lower lung burden, thus adding to the inflammation. The concomitant disruption of efferocytosis (clearance of apoptotic cells via phagocytosis) creates a delayed “secondary” necrosis (Hodge et al., 2003; Oberdorster, 1995; Schmidt and Tuder, 2010). Unlike apoptosis (programmed cell death), which controls potentially damaging inflammation by not releasing the contents of the cell into the surrounding environment, necrotic cell death adds to the burden on the body and can have a negative cascading effect (Nanji and Hiller-Sturmhofel, 1997; Schmidt and Tuder, 2010). Therefore, while exposure to reactive foreign material alone can also generate necrotic cell death (Harrington et al., 2012b), inhibition or reduced efficiency in efferocytosis exacerbates the problem.

A number of toxicity markers can be utilized to evaluate the effect of particle exposure on lung cells (Antonini et al., 2009; Chamnongpol et al., 2002; Gloyne et al., 2011; Kida et al., 2005). The approach developed by Harrington et al. (2012) takes into account the possible complexity of immune responses to foreign materials in the body by capturing the immediate and short-term inflammatory stress response (ISR) generated by exposure to a contaminant (Harrington et al., 2012b). The ISR is the normalization of the cellular upregulation of ROS with respect to the cell viability. It not only allows for overall stress levels to be evaluated but each component as well. Due to the relatively short time course of the exposure, the cell viability is a measure of necrotic cell death. Whereas the amount of cellular ROS upregulation is not only a marker of immediate cellular stress, but also may indicate future apoptotic cell death (Fleury et al., 2002; Yoo et al., 2012).

By focusing on the ISR, a better understanding of the inflammatory nature of a material can be established, although the specific biological pathways interrupted by the material cannot be resolved without additional studies. In this study, the protocol developed by Harrington and coworkers is used to compare the ISR for eight common metal-sulfide ore minerals. The results allow for direct comparison between materials, which is the basis for an evaluation on whether the structure and chemistry of (earth) materials are important determining factors in toxicity.

The cell culture work in this study is complemented by an assessment of the generation of $\cdot\text{OH}$ and H_2O_2 by the metal-sulfides when dispersed in water in the absence of an epithelial lung cell line. This acellular test provides insight in purely mineral-driven ROS formation, which can be useful in the interpretation of ISR data. It is hypothesized that the ability of metal-sulfide minerals to generate ROS when dispersed in water will contribute to an elevated ISR. Furthermore, we expect that minerals containing ferrous iron, and to a lesser extent monovalent copper, will produce more ROS when dispersed in water as a result of their ability to promote the Fenton reaction (eq. 1). It should be pointed out, however, that materials that do not form ROS when dispersed in water, e.g. glass beads, will elicit a modest cellular upregulation of ROS within epithelial cells (Harrington et al., 2012b). This is the result of the immune response of epithelial cells to any foreign substance. Metals released from the mineral particles may also lead to interference of biochemical processes in the cell and contribute to a loss of cell viability (Valko et al., 2005), which would lead to higher ISR values independent of the ability of the mineral to generate ROS when dispersed in water. The completion of this study will allow for a better understanding of the inflammatory nature of a number of common metal-sulfide ore minerals.

2. Materials and Methods

2.1. Mineral sample preparation

Eight natural metal-sulfide ore minerals, obtained from Wards (Rochester, NY), were investigated. The samples include three zinc sulfides with variable iron contents, one copper sulfide, two copper iron sulfides, a lead sulfide, and an iron arsenic sulfide (Table 1). All samples were ground and sieved to obtain a suitable respirable fraction. The materials were characterized using a suite of complementary techniques.

The mineralogical composition of samples was determined by X-Ray Diffractometry (XRD) using a Scintag PAD X diffractometer under the following conditions: $\text{CuK}\alpha_1$, 40kV, 25 mA, $5^\circ - 75^\circ 2\theta$, step $0.02^\circ 2\theta$ and counting times of 5-10 seconds per step. The mineralogy of the materials used in this study is summarized in Table 1. Trace metal composition of the materials was determined using X-ray Fluorescence (XRF). An aliquot of the ground material was pressed into an 18mm tablet with high-purity cellulose as binder. The XRF analysis was conducted on a Bruker Pioneer wavelength-dispersive XRF spectrometer in standardless mode, which means that the XRF intensities were converted to concentrations on the basis of a physical X-ray absorption model provided with the instrument. The XRF results are summarized in Table 2 along with nominal major element concentrations based on ideal mineral compositions. It is important to note that the pressed tablet method is not suitable for major element determination, hence the inclusion of nominal major element concentrations in this table.

Specific surface area was determined for each material, because the exposure experiments are normalized with respect to surface area. Specific surface area (m^2/g) of each sample after grinding was determined using gas adsorption measurements. The measurements were conducted on a Quantachrome NOVA 2200e BET instrument, employing a 5-point BET isotherm ($r^2 = 0.999$), with Ultra-High Purity N_2 as adsorbate. The specific surface area of all metal-sulfides evaluated in this study are summarize in Table 1.

2.2. Acellular Hydroxyl radical and Hydrogen Peroxide Formation

The formation of $\cdot\text{OH}$ and H_2O_2 was determined using the protocol developed by Cohn et al. (2009) (Cohn et al., 2009). The protocol is based on the conversion of non-fluorescent 3'-(p-Aminophenyl) fluorescein (APF) to fluorescein, which is strongly fluorescent. The APF probe reacts with $\cdot\text{OH}$ but not with H_2O_2 ; however, with the addition of horseradish peroxidase (HRP), H_2O_2 becomes reactive and can be quantified using APF. Hence, by conducting two complementary experiments, one without HRP and one with HRP, both $\cdot\text{OH}$ and $\cdot\text{OH} + \text{H}_2\text{O}_2$ formation can be determined. It is important to note that the APF probe records the cumulative formation of either $\cdot\text{OH}$ (without HRP) or $\cdot\text{OH} + \text{H}_2\text{O}_2$ (with HRP) over the duration of the experiment. Hence, the APF protocol does not provide an analysis of the concentration of these ROS at any given time during the experiment. Furthermore, fluorescein formed in the experiment will be (partially) degraded if the production of either $\cdot\text{OH}$ or H_2O_2 exceeds the available amount of APF in the experiment. Therefore, insufficient amounts of APF probe in the experiment will lead to an underestimation of the amount of $\cdot\text{OH}$ or $\cdot\text{OH} + \text{H}_2\text{O}_2$ formed.

Two sets of experiments with APF were conducted. In a first set, an appropriate amount of each of the sulfides were weighed into 3 mL polyethylene snap-top tubes to provide a surface loading of about $10 \text{ m}^2/\text{L}$ due to differences in surface area (masses weighed can be calculated using the surface areas provide in Table 1; ie. 0.0633g and 0.0315g for chalcopyrite and arsenopyrite, respectively). Water kept in an amber plastic bottle for at least 30 days to minimize background H_2O_2 levels was added to the minerals by micropipette (2964 μL in experiments without HRP or 2844 μL in experiments with HRP). Subsequently, 30 μL of 5 M phosphate buffer was added, followed by 6 μL of APF. In the

experiments with HRP, 120 μL of HRP was added as a final reagent. The tubes were capped, placed on a vortex mixer for 15 second to mix the reagents, and then transferred to a Labquake orbital shaker, which continuously mixed the mineral slurries by rotating end-over-end. The entire orbital shaker was kept in the dark to prevent photochemical degradation of the APF probe or fluorescein. After 26 hours of mixing, the tubes were removed from the orbital shaker, filtered over Teflon micro-membrane filters with a pore size of 0.22 μm , and the fluorescence was measured on a Turner Design instrument, equipped with 490 (excitation) and 520 (emission) filters. A calibration curve made by diluting a 1 mM H_2O_2 stock solution and reacting it with APF and HRP (Cohn et al., 2009). The fluorescence values recorded for the mineral slurries was converted to H_2O_2 equivalents and expressed as nM/m^2 .

Three minerals, chalcopyrite, galena, and arsenopyrite were studied further by conducting a series of shorter duration experiments to determine the evolution of $\cdot\text{OH} + \text{H}_2\text{O}_2$ over time. In these experiments, the volume of APF probe molecule added was increased to 12 μL and the amount of water added was lowered by 6 μL . The volumes of buffer and HRP remained the same. The same volume adjustments were made for the experiments to determine the calibration curve. The additional volume of APF was needed to prevent the probe molecule from becoming exhausted as a result of high amounts of $\cdot\text{OH} + \text{H}_2\text{O}_2$ formed in some experiments.

2.3. Culturing and Plating the A549 Human Lung Epithelial Cell Line

A previously published protocol was utilized for the culture of the A549 human lung epithelial cell line (Harrington et al., 2012b). The cell were maintained in 10% Fetal Bovine Serum (FBS) and 1% 1X Penicillin/Streptomycin in Ham's F12K Media. Cell work was performed in a sterile hood. The cells were kept in an incubator at standard conditions (37°C, 95% and 5% CO_2 in air, respectively). For passage and counting purposes, once confluent, the cells were lysed using trypsin with ethylenediaminetetraacetic acid (EDTA). Trypan blue was the staining agent used in conjunction with a hemocytometer for cell quantification. Cells were loaded into 96-well microplates at 8×10^4 cells/mL. However, columns 1, 2, 11, and 12 were kept cell free for normalization purposes. When not in the hood, a lid was kept on the microplate.

2.4 Inflammatory Stress Response Measurements

To determine the ISR generated in the A549 cells by mineral samples a protocol developed by Harrington et al. was used (Harrington et al., 2012b). In brief, 2',7'-dichlorofluorescein-diacetate (DCFH-DA) assay from Sigma Aldrich and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) from Promega were utilized concomitantly for cellular ROS upregulation and viability quantification, respectively. All mineral slurries were normalized to surface area with the stock solution containing 0.002 m^2 mineral contaminant/mL Hank's Buffered Salt Solution (HBSS). Each experiment was replicated by using two identically prepared 96-well plates; therefore for each mineral contaminant there were eight wells for each particle loading allowing for a robust data set. Particle loadings were elevated to highlight differences in reactivity and do not necessarily represent typical exposure concentrations. Given ambient air and

occupational health standards, the lower loadings utilized in the study (i.e. 0.00025 m²/mL) better represent actual exposure concentrations (Aneja et al., 2012; George et al., 2013).

The first analysis occurred after a 30 minute exposure, followed by 1 hour, 2 hours, 4 hours, 8 hours and 24 hours. ROS upregulation measurements were made using Thermo Scientific's Fluoroskan Ascent and cell viability measurements were made on a Molecular Devices' SpectraMax 340PC384, since DCFH-DA is a fluorometric assay, while MTS is a colorimetric probe. The microplates were covered with foil during transport to minimize exposure to light.

3. Results

The metal-sulfides studied here show a wide range of results in terms of both acellular ROS formation and ISR. The results of the acellular and cellular work are presented in two separate sections for clarity.

3.1. Acellular ROS formation

Chalcopyrite, galena, and arsenopyrite show significant amounts of $\cdot\text{OH} + \text{H}_2\text{O}_2$ formation when dispersed in water compared to the other sulfides studied (Table 3). In fact, upon further investigation, the $\cdot\text{OH} + \text{H}_2\text{O}_2$ values for chalcopyrite are actually an underestimate due to APF probe exhaustion (Figure 1). The extreme $\cdot\text{OH} + \text{H}_2\text{O}_2$ production of chalcopyrite coincides with it generating the highest amount of $\cdot\text{OH}$ and contrasts with the sphalerite sample from China which did not produce high enough fluorescence signals to quantify $\cdot\text{OH} + \text{H}_2\text{O}_2$ let alone $\cdot\text{OH}$. There was also no quantifiable $\cdot\text{OH} + \text{H}_2\text{O}_2$ production from the sphalerite sample from Spain. The sphalerite sample from the United States recorded very low values for both $\cdot\text{OH}$ and $\cdot\text{OH} + \text{H}_2\text{O}_2$ despite its high iron content.

The results of a second set of experiments with chalcopyrite, galena, and arsenopyrite in which the amount of APF was increased and the reaction time shortened are presented in Figure 1. The results with chalcopyrite showed that the production of $\cdot\text{OH} + \text{H}_2\text{O}_2$ started immediately and led to the generation of μM levels of $\cdot\text{OH} + \text{H}_2\text{O}_2$ within an hour. By contrast, the buildup of $\cdot\text{OH} + \text{H}_2\text{O}_2$ was slower in galena and arsenopyrite slurries and the levels attained were well below those attained in chalcopyrite slurries. These data indicate that chalcopyrite, when dispersed in water, will trigger a burst of H_2O_2 , a part of which will convert to $\cdot\text{OH}$ (Table 3).

3.2. Cellular Response

The ISR and cell viability data of all eight natural metal-sulfide ore minerals are presented in Figure 2. The three different sphalerite samples had little or no effect on cell viability and generated low levels of ROS upregulation, resulting in low ISR values. In fact, their response was comparable to glass beads and anatase, which are considered relatively inert or unreactive (Table 4). By contrast, the copper sulfide minerals bornite, chalcopyrite, and to a lesser extent chalcocite showed a significant loss in cell viability combined with ROS upregulation in the remaining viable cells, leading to high ISR values. Galena also showed a significant loss of cell viability, however due to low ROS upregulation the ISR values were comparable to those of chalcocite. Arsenopyrite generated a modest loss of viability

combined with little ROS upregulation, as a result the ISR values were comparable with materials that are considered inert or unreactive, such as glass beads and anatase. The experimental design allows for not only a range of particle loadings to be evaluated but, by taking reading between 30 minutes after exposure to 24 hours, it is possible to evaluate the changes in ISR over time. These temporal variations as well as more details for each type of metal-sulfide are presented below. To place the results obtained in this study into context, the results were compared to standard reference materials as well as relatively inert material and pyrite that have been evaluated using the same protocol (Harrington et al., 2012b), see Table 4. Below, the results for each of the materials tested are described. Unless otherwise stated, the ISR values mentioned below refer to those associated with experiments at the highest particle loading ($0.002 \text{ m}^2/\text{mL}$). The statistics for Figures 3-5 can be found in the supplementary material.

3.2.1. Zinc Sulfides—The three sphalerite samples contain different amounts of iron (Spain-Picos de Europa, 0.154 wt. %; China-Yu Chen, 0.189 wt. %; and USA-Balmat, 6.603 wt. %) and represent a compositional variation that is well within the range of iron content recorded for natural sphalerite. Overall, as mentioned above, these three sphalerite samples do not elicit a strong inflammatory response, nor do they elicit a significant sustained loss of cell viability. However, they do show a difference in how they respond through time (Figure 3). The maximum ISR occurred at 1 hour for the Yu Chen sample, compared to 4 hours for the Picos de Europa sample, and 24 hours for the Balmat sample. Of the three sphalerite samples, the sample from Picos de Europa generated the greatest ISR of the sphalerite samples at 362 % of the control. Given that the cell viability does not drop below 85%, the ISR is mostly a result of the upregulation of ROS within the cells. However, the upregulation is modest compared to the copper sulfides and on par with that seen for glass beads.

3.2.2. Copper Sulfides—Chalcocite, the only iron-free copper sulfide investigated, generated the lowest ISR of the group at 3,408 % of the control. This high stress response was caused by both a drop in cell viability (39 % of the control at 24 hours) and a high ROS upregulation. Chalcopyrite, the most reactive mineral of the copper sulfides and all metal-sulfides tested here, generated an ISR of 86,024 % of the control at the 2 hour time point. However, by 24 hours the ISR decreased to 27,370 % of the control (Figure 3). While there was significant ROS upregulation, these ISR values are driven to extremes given the low cell viabilities (1.7 % of the control at 2 hours and 3.3 % at 24 hours). Bornite, generating an ISR value of 40,918 % of the control at the 8 hour time point, also resulted in extremely low viability (2.8 % of the control). Since exceedingly low cell viabilities at the highest particle loadings distort the ISR values, it is more meaningful to focus on the response at lower loadings. All copper sulfides cause high ISR values under moderate ($0.001 \text{ m}^2/\text{mL}$) particle loadings (Figure 4). The inflammatory stress generated by chalcocite and bornite increased with time, generating ISR values of 1,079 and 1,727 % of the control, respectively; while the toxicity of chalcopyrite decreased with time (3,168 % of the control at 30 minutes and 888 % of the control after 24 hours).

3.2.3. Lead Sulfide—Galena generated a maximum ISR after 30 minutes of exposure (3,194 % of the control for highest loading). This ISR was caused by the immediate and dramatic drop in cell viability (down to 3.5 % of the control), while the ROS upregulation remained essentially unaffected (110 % of the control). After this initial high ISR value, a steady decrease in ISR was observed over the first 8 hours period as a result of recovery of cell viability (27 % of the control at 8 hours). However, at 24 hours the ISR increased slightly (405 % of the control) with the decrease in cell viability (20 % of the control). Given the low viability of the cells at the 30 minutes time point at the highest particle loading, the moderate particle loading was also examined. With a cell viability of 73 % of the control and an ROS upregulation of 115 % of the control, the ISR was only at 157 % of the control. However, the viability decreased to 44 % of the control at 24 hours.

3.2.4. Iron Arsenic Sulfide—The arsenopyrite tested here elicited only a modest inflammatory response. The response was more pronounced than that for the three sphalerite samples but comparable with a NIST San Joaquin Valley soil with baseline trace metal contents (Table 4). The highest ISR generated by the arsenopyrite was 476 % of the control at 24 hours. The ISR did not increase steadily with time. The ROS upregulation and cell viability consistently decreased with increasing exposure time. The viability ranged from 102 % of the control at 30 minutes and 37 % of the control at 24 hours. Under moderate particle loading, the ISR generated by arsenopyrite was slightly higher than that generated by galena, although the cell viability was nearly twice as high (73 % of the control) at 24 hours.

4. Discussion

The metal-sulfide ore minerals investigated in this study generated a wide range of ISR values and only chalcopyrite produced both substantial amounts of H_2O_2 and $\cdot\text{OH}$ when dispersed in water. Since the ISR is derived by normalizing the cellular upregulation of ROS by the cell viability, it can be elevated by not only high ROS upregulation but also low viability. While this study focuses on evaluating ISR generated by the exposure to common ore minerals, the origin of the stress is important because it can point to different mechanisms by which exposure to these types of materials may affect human health. Furthermore, understanding the type of cell death generated can make a difference in treatment (Nanji and Hiller-Sturmhofel, 1997).

The iron-copper sulfides elicit by far the most pronounced cellular response, followed by copper sulfide. The recorded ISR values are predominately driven by necrotic cell death. The loss of cell viability generated by bornite and chalcopyrite is so immediate and resounding at the highest particle loading ($0.002 \text{ m}^2/\text{mL}$) that the cells are not able to recover at all during the 24 hour period (Figure 2). In fact the ISR values for chalcopyrite are higher than those seen in experiments with pyrite that was not acid washed (Table 4) (Harrington et al., 2012b). When experiments with moderate particle loadings ($0.001 \text{ m}^2/\text{mL}$) of chalcopyrite or bornite are considered, the viability among cells exposed to chalcopyrite actually increases with time (from 17% to 78% confluence), while the viability of cells exposed to bornite stays relatively stable ($75 \pm 7\%$). In other words, at the $0.001 \text{ m}^2/\text{mL}$ loading level, exposure to chalcopyrite leads to a significant immediate loss of cell

viability followed by a rebound, while such an effect is not seen in the experiments with bornite.

It is possible that the difference in cell viability is related to the spontaneous generation of ROS by chalcopyrite when dispersed in water (Figure 1). Studies show a cathodic interaction between the copper and iron in chalcopyrite, allowing for increased ROS production compared to pyrite alone (Kaur et al., 2013; Kaur and Schoonen, (submitted)). The increased ability to take part in Fenton chemistry might increase the toxicity of chalcopyrite. Unlike chalcopyrite, bornite shows little or no ROS generation when dispersed in water (Table 3). Although low cellular viabilities, particularly at the highest concentration, drive up the ISR values, the chalcopyrite and bornite particles also generate a strong upregulation of ROS within the cells. This indicates that the remaining cells are likely in the early stages of apoptosis. At a loading of 0.001 m²/mL, the ISR values for bornite in the first 4 hours are similar to those determined for chalcocite, galena, and arsenopyrite (Figure 4). After 8 to 24 hours, there is no longer a significant difference in ISR values between bornite and chalcopyrite (Figure 3 and 4), suggesting that the toxicity of bornite is related to a slower process than ROS generation upon dispersion in water. One possibility is that it takes a few hours for copper and iron released from the bornite surface to build up to concentration levels that start to impair biological functions in the cells. In fact, after 24 hours the ISR of bornite was higher at a loading of 0.001 m²/mL than that of chalcopyrite, and the ISR of chalcocite was comparable with that of chalcopyrite (demonstrated a similar trend to bornite). If the ISR is compared for low particle loadings (0.00025 m²/mL), that are more relevant to actual single exposure levels (Aneja et al., 2012; George et al., 2013), the effect of cell death is negligible. At this lower loading the ISR generated by bornite was the greatest, followed by chalcocite and chalcopyrite (Figure 5). These results are not unexpected since at the higher loadings the ISR generated by bornite and chalcocite are predominately increased by an upregulation of ROS within the cells. The ISR derived by chalcopyrite, on the other hand, is predominately driven by cell death. While the Fenton reaction is often presented with iron as the metal, copper is also a Fenton metal (Strlic et al., 2003; Valko et al., 2005) and it may also interfere with other processes in the cell. The simultaneous release of copper and iron leads to particularly high ISR as exemplified by chalcopyrite and bornite at the highest loading level and bornite and the 0.001 m²/mL loading level.

After the copper sulfides, galena generated the highest ISR. However, galena exhibits an immediate spike in ISR driven by a drastic loss in cell viability, followed by a recovery of the system and lower ISR values. This temporal trend, which is very different from the other sulfides studied here, may be due to a change in surface properties over time or the initial generation of $\cdot\text{OH} + \text{H}_2\text{O}_2$ (Figure 1). Oxidation products of galena are relatively insoluble and due to the increase in surface coating that occurs during the dissolution, the rate of dissolution decreases with time (De Giudici and Zuddas, 2001). It must be noted that anglesite, the lead sulfate dissolution product, is toxic if allowed to accumulate in the body. However, since it is a cumulative poison, the small amount of anglesite that may be present in this study would not be expected to cause damage. The ISR generated by galena can be attributed to extensive necrotic cell death. The upregulation of ROS stays predominantly

stable when compared to control wells and even decreases with time. This trend in behavior is interesting, given the overall increase in viability with time (3.5% to 20%).

The ISR values generated by arsenopyrite are perhaps surprisingly low given that the mineral contains ferrous iron and generates H_2O_2 when dispersed in water (Figure 1). It is possible that the dissolution and release of iron is limited in the more complex fluids used in the cellular studies, similar to earlier results with pyrite, which showed a reduction in dissolution rate (Harrington et al., 2012a). Furthermore, at a neutral pH, ferric iron-hydroxide coatings dominate the surface of arsenopyrite and sulfur, not iron, is readily released into solution (Asta et al., 2010). Although the ISR for arsenopyrite is low and falls within the range generated by relatively inert materials (e.g. glass bead and anatase, Table 4), the cell viability data indicates that arsenopyrite does affect epithelial cells.

Characteristic response of cells exposed to relatively inert material (e.g., glass beads) is to upregulate ROS and maintain a steady, if not increasing, cell viability when compared to a control (Harrington et al., 2012b). However, the cell viability data for arsenopyrite indicates significant necrotic cell death after 24 hours (Figure 2). Unlike glass beads, arsenopyrite does not upregulate ROS; hence, the resulting ISR value is low. A similar pattern is observed in a study utilizing small cell lung carcinoma (SCLC) cells, with arsenic generating predominately necrotic and not apoptotic cell death (Pettersson et al., 2009). This divergent behavior is similar to that seen by galena, where the ISR is driven up primarily by cell death and not by a cellular upregulation of ROS. Unlike other metals in this study, both arsenic and lead are known immunosuppressants which might explain the limited upregulation of ROS (Klaassen and Watkins, 2010; Roussel and Barchowsky, 2000).

The ISR generated by the sphalerite samples are the lowest among the sulfide mineral studied here. The ISR among the sphalerite samples generally decreased with increasing iron content, which suggests that Fenton-driven chemistry with iron does not play a role. In fact, the presence of structural iron appears to limit the cytotoxicity. As reported for iron-coated quartz (Ghiazza et al., 2011), a large amount of structural iron within the sphalerite may lead to the formation of a ferric oxide coating that may render the material less cytotoxic. The iron oxyhydroxide patches also limit the dissolution rate. Similar to arsenopyrite, the ISR generated by all the sphalerite samples fall within the range generated by inert material (Harrington et al., 2012b). However, unlike arsenopyrite, for the most part the ISR values are driven by an upregulation of ROS. Trace metals other than iron, such as mercury, may also play a role in the differentiation between the three sphalerite samples. Unlike any of the other sulfides tested here, the sample of Picos de Europa contained measurable amounts of mercury (1300 ppm). Furthermore, it is important to note that although the Balmat sphalerite did not generate a high ISR, the cell viability did decrease with time (95% versus 82%). A decrease in viability over time is not seen in inert material and indicates the inflammation generated by the Balmat sphalerite is likely to continue to increase with time.

5. Conclusions

The ISR is used to evaluate the toxicity of ore and/or associated ore minerals. Except in the case of chalcopyrite, there is no clear correlation between the metal-sulfides ability to generate ROS when dispersed in water and generate an ISR. The minerals containing ferrous

iron and copper generated the greatest ISR values, driven by both an upregulation of ROS and necrotic cell death. It is possible the interaction between iron and copper magnifies the toxicity of a material. Minerals containing lead, arsenic and ferric iron generated ISR values based on pronounced necrotic cell death combined with minimal ROS upregulation. The importance of evaluating the ISR and its individual components is highlighted in this study. While the actual biological pathways interrupted are unknown, understanding the inflammatory response generated by a material and the type of cell death that results is important in determining not only the deleterious nature of the material but also a first step in developing a better understanding of pathogenesis and designing possible treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Kinetic analysis of reactive metal-sulfide ore minerals

Fluorescence measurements quantifying $\text{H}_2\text{O}_2 + \cdot\text{OH}$ production in an acellular system (phosphate buffered solution at a pH of 7.4) taken as a function of time for galena, arsenopyrite and chalcopyrite are represented. Material loading was at $10 \text{ m}^2/\text{L}$.

Figure 2. ISR of eight metal-sulfide ore minerals

The ISR of the eight study materials are represented, with cell viability for each in the inset.

(a) Sphalerite – Yu Chen Mine (China), (b) sphalerite – Balmat, New York (USA), (c) sphalerite – Picos de Europa (Spain), (d) arsenopyrite, (e) galena, (f) chalcocite, (g) bornite, and (h) chalcopyrite. For some data points, the error bars (representing standard deviation of eight replicates) are obscured by the symbols.

Figure 3. Evolution of ISR over time for the highest particle loadings

The ISR generated over time by all the metal-sulfide ore minerals tested at the highest particle loading ($0.002 \text{ m}^2/\text{mL}$) is represented. For some data points, the error bars (representing standard deviation of eight replicates) are obscured by the symbols.

Figure 4. Evolution of ISR over time for the moderate particle loadings

The ISR generated over time by all the metal-sulfide ore minerals tested at the moderate particle loading ($0.001 \text{ m}^2/\text{mL}$) is represented. For some data points, the error bars (representing standard deviation of eight replicates) are obscured by the symbols.

Figure 5. Evolution of ISR over time for low particle loadings

The ISR generated over time by selected metal-sulfide ore minerals tested at a low particle loading ($0.00025 \text{ m}^2/\text{mL}$) is represented. For some data points, the error bars (representing standard deviation of eight replicates) are obscured by the symbols.

Table 1

Metal-sulfide mineral ores

	Mineral	Locality	Accessory Minerals	Surface Area (m ² /g)
Zinc Sulfides	Sphalerite	Picos de Europa, Spain	Trace unknown, no pyrite or quartz	0.368
	Sphalerite	Yu Chen Mine, China	Trace unknown, no pyrite or quartz	0.345
	Sphalerite	Balmat, NY USA	Trace quartz	1.102
Copper Sulfides	Chalcocite	Butte, MT USA	Minor quartz, trace djurleite ^a	1.045
	Chalcopyrite	Durango, Mexico	Trace quartz	0.474
	Bornite	Magma Mine, AZ USA	Trace chalcopyrite, djurleite	0.654
Lead Sulfide	Galena	Tri State Mining District, MO-KS-OK USA	none	0.165
Iron Arsenic Sulfide	Arsenopyrite	Panasqueira, Mexico	Minor marcasite	0.952

^aThe chemical formula of djurleite is Cu₃1S16

Table 2

Trace metal concentration of metal sulfides used in study^a

Mineral	Formula	As	error	Cu	error	Fe	error	Hg	error	Mn	error	Pb	error	Zn	error
Arsenopyrite	FeAsS	<i>460,100</i>	na.	679	3.07	<i>343,000</i>	na.	<DL		<DL		<DL		<DL	
Bornite	Cu ₃ FeS ₄	<DL	n.a.	633,100	n.a.	<i>113,000</i>	na.	<DL		<DL		<DL		<DL	
Chalcopyrite	CuFeS ₂	830	7.92	<i>346,300</i>	n.a.	<i>304,300</i>	na.	<DL		120	8.97	579	5.50	8,772	0.55
Chalcocite	Cu ₂ S	<DL	n.a.	<i>798,500</i>	n.a.	<i>52,700</i>	0.21	<DL		<DL		<DL		2,390	1.00
Galena	PbS	<DL	n.a.	1,150	2.71	1,020	2.35	<DL		<DL		86.6	na.	<DL	
ZnS, USA	ZnS	<DL	n.a.	<DL	n.a.	<i>66,000</i>	0.20	<DL		605	3.08	<DL		<i>640,600</i>	n.a.
ZnS, Spain	ZnS	<DL	n.a.	<DL	n.a.	1,540	1.46	1,300	4.89	n.d		550		<i>640,600</i>	n.a.
ZnS, China	ZnS	<DL	n.a.	<DL	n.a.	1,890	1.30	<DL		n.d		842	4.97	<i>640,600</i>	n.a.

^a All concentrations in ppm with major constituents italicized. Trace element data determined on pressed pellets using semi-quantitative data evaluation method. Error given in %; <DL = below the detection limit, n.a.=not applicable. See text for details.

Table 3ROS formation by selected metal sulfides in acellular slurries^a

Mineral	OH* (nM/m ²)	OH + H ₂ O ₂ (nM/m ²)
Chalcocite	2	14
Chalcopyrite	41	477 ^b
Bornite	1	4
Arsenopyrite	12	448
Galena	6	307
Sphalerite_Picos de Europa	<DL	1
Sphalerite_Yu Chen	<DL	<DL
Sphalerite_Balmat	2	5

<DL = below the detection limit

^aCumulative amounts of OH and H₂O₂ recorded after 26 hour incubation period.^bUnderestimate due to APF probe exhaustion.

Table 4

Compilation of ISR studies for comparison

	Material	Time to highest ISR ^a	Cellular ROS Upregulation ^b	Cellular Viability ^c	Inflammatory Stress Response ^d
Synthetic	Anatase ^e	M	+	xx	++
	Glass Beads ^e	S	+	o	++
Soil	San Joaquin Valley ^e	S	+	xx	+
	Montana ^e	L	++	xxx	++++
Mineral	Sphalerite (China)	S	+	x	+
	Sphalerite (USA)	L	+	xx	+
	Sphalerite (Spain)	M	+	x	++
	Arsenopyrite	L	+	xxxx	+++
	Galena	S	+	xxxxxx	++++
	Chalcocite	L	++++	xxxx	++++
	Bornite	M	++++	xxxxxx	+++++
	Pyrite ^e	L	+++	xxxxxx	+++++
	Chalcopyrite	M	++++	xxxxxx	+++++
	Pyrite (Acid-Treated) ^e	M	++++	xxxxxx	+++++

^aExposure High at: 0-1 hr (S)hort; 2-8hrs Inter(M)ediate; 24hs (L)ong^bIf % of control: 100-450 +; 451-600 ++; 601-1,000 +++; >1,001 ++++^cIf % of control: >101 o; 100-85 x; 84-70 xx; 69-50 xxx; 49-30 xxxx; 29-10 xxxxx; <9 xxxxxx^dIf % of control: 100-300 +; 301-450 ++; 451-1,000 +++; 1,001-4,000 ++++; 4,001- 10,000 +++++; 10,000 -50,000 ++++++; 50,001-100,000 ++++++; >100,000 ++++++^eFrom Harrington et al. (2012)