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Use of Electron Paramagnetic Resonance (EPR) in Biological Samples at Ambient Temperature and 77 K

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TITLE:

Use of Electron Paramagnetic Resonance in Biological Samples at Ambient Temperature and 77 K

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KEYWORDS:

Electron paramagnetic resonance, spin probes, CMH, CPH, mito-TEMPO-H, superoxide, reactive oxygen species, nitroxide, superoxide dismutase, polytetrafluoroethylene tubing

SUMMARY:

Electron paramagnetic resonance (EPR) spectroscopy is an unambiguous method to measure free radicals. The use of selective spin probes allows for detection of free radicals in different cellular compartments. We present a practical, efficient method to collect biological samples that facilitate treating, storing, and transferring samples for EPR measurements.

ABSTRACT:

The accurate and specific detection of reactive oxygen species (ROS) in different cellular and tissue compartments is essential to the study of redox-regulated signaling in biologicalsettings. Electron paramagnetic resonance spectroscopy (EPR) is the only direct method to assess free radicals unambiguously. Its advantage is that it detects physiologic levels of specific species with a high specificity, but it does require specialized technology, careful sample preparation, and appropriate controls to ensure accurate interpretation of the data. Cyclic hydroxylamine spin probes react selectively with superoxide or other radicals to generate a nitroxide signal that can be quantified by EPR spectroscopy. Cell-permeable spin probes and spin probes designed to

accumulate rapidly in the mitochondria allow for the determination of superoxide concentration in different cellular compartments.

In cultured cells, the use of cell permeable 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine (CMH) measures total nitroxide signal. This probe is highly selective for superoxide and does not react with hydrogen peroxide. The treatment of samples with and without cell-impermeable superoxide dismutase (SOD) pretreatment, or use of cell-permeable PEG-SOD, then allows for the differentiation of extracellular from cytosolic superoxide. The mitochondrial 1-hydroxy-4-[2-triphenylphosphonio)-acetamido]-2,2,6,6-tetramethyl-piperidine,1-hydroxy-2,2,6,6-tetramethyl-4-[2-(triphenylphosphonio)acetamido] piperidinium dichloride (mito-TEMPO-H) allows for measurement of mitochondrial ROS (predominantly superoxide).

Spin probes and EPR spectroscopy can also be applied to *in vivo* models. Superoxide can be detected in extracellular fluids such as blood and alveolar fluid, as well as tissues such as lung tissue. Several methods are presented to process and store tissue for EPR measurements and deliver intravenous 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine (CPH) spin probe *in vivo*. While measurements can be performed at room temperature, samples obtained from *in vitro* and *in vivo* models can also be stored at -80 °C and analyzed by EPR at 77 K. The samples can be stored in specialized tubing stable at -80 °C and run at 77 K to enable a practical, efficient, and reproducible method that facilitates storing and transferring samples.

INTRODUCTION:

While measures of oxidative stress and reactive oxygen species are important to the study of diverse diseases across all organ systems, the detection of reactive oxygen species (ROS) is challenging due to a short half-life and high reactivity. An electron paramagnetic resonance (EPR) technique is the most unambiguous method for detecting free radicals. Spin probes have advantages over the more commonly used fluorescent probes. Though fluorescent probes are relatively inexpensive and easy to use and provide rapid, sensitive detection of ROS, they do have serious limitations due to artifactual signals, an inability to calculate ROS concentrations, and a general lack of specificity[1].

To facilitate the use of EPR for biologicalstudies, a variety of spin probes have been synthesized that can measure a range of biologically relevant free radical species as well as pO_2, pH, and redox states[2-7]. Spin traps have also been developed to capture short-lived radicals and form long-living adducts, which facilitates detection by EPR[8]. Both classes (spin probes and spin traps) have advantages and limitations. One commonly used class of spin probes are cyclic hydroxylamines, which are EPR-silent and react with short-lived radicals to form a stable nitroxide. Cyclic hydroxylamines react with superoxide 100 times faster than spin traps, enabling them to compete with cellular antioxidants, but they lack specificity and require the use of appropriate controls and inhibitors to identify the radical species or source responsible for the nitroxide signal. While spin traps exhibit specificity, with distinct spectral patterns depending on the trapped species, they have slow kinetics for superoxide spin trapping and are prone to

biodegradation of the radical adducts. Applications for spin trapping have been well-documented in biomedical research[9-13].

The goal of this project is to demonstrate practical EPR methods for designing experiments and preparing samples to detect superoxide using spin probes in different cellular compartments *in vitro* and in different tissue compartments *in vivo*. Several manuscripts have published protocols relevant to these goals, using cell-permeable, cell-impermeable, and mitochondrial targeted spin probes to target different cellular compartments *in vitro* and process tissue for analysis in mouse models[14,15]. We build upon this body of literature by validating an approach to measure superoxide using a 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine (CMH) spin probe in different cellular compartments *in vitro* to ensure accurate measurements, highlighting potential technical problems that may skew results. We also provide methods to perform EPR measurements in blood, bronchoalveolar lavage fluid, and lung tissue using the CMH spin probe. These studies compare different methods to process the tissues as well as present a method to inject another spin probe, CPH, into mice prior to harvesting tissue. Finally, we develop a practical method to store samples in polytetrafluoroethylene (PTFE) tubing to allow for the storage and transfer of samples before EPR measurements at 77 K.

PROTOCOL:

All animal studies were approved by the University of Colorado Denver Institutional Animal Care and Use Committee.

1. Preparation of Reagents

1.1. Diethylenetriaminepentaacetic acid (DTPA) stock (150 mM)

1.1.1. Add 2.95 g of DTPA (393.35 g/mol) to 10 mL of deionized water.

1.1.2. To dissolve DTPA, add 1 M NaOH dropwise and bring to a pH of 7.0.

1.1.3. Bring the volume to 50 mL with water for a final DTPA concentration of 150 mM, and store at 4 °C.

1.2. Phosphate buffer saline (PBS) (50 mM, pH 7.4)

1.2.1. Prepare 5 M of sodium chloride (NaCl) (58.44 g/mol; 29.22 g/100 mL).

1.2.2. Prepare 1 M of potassium phosphate dibasic HK_2PO_4 (174.18 g/mol; 17.42 g/100 mL)

1.2.3. Prepare 1 M of potassium phosphate monobasic KH_2PO_4 (136.1 g/mol; 13.61 g/100 mL). Mix 3 mL of 5 M NaCl with 4.24 mL of 1 M potassium phosphate dibasic and 0.760 mL of 1 M potassium phosphate monobasic. Check the pH.

1.2.4. Bring the volume to 100 mL with deionized water.

1.2.5. Store at room temperature (RT) for short-term (days) and at 4 °C for long-term (weeks) storage.

1.3. Krebs-Henseleit buffer (KHB) containing 100 µM DTPA

1.3.1. In 50 mL conical centrifuge tube, add 33.3 µL of 150 mM DTPA stock solution.

1.3.2. Bring to a 50 mL volume with Krebs-Henseleit buffer (KHB).

1.3.3. Prepare fresh buffer with DTPA each day and keep it at RT.

1.4. Tris-EDTA buffer containing sucrose

1.4.1. Prepare 0.5 M Tris stock: dissolve 15.14 g of Tris base (121.14 g/mol) in 150 mL of deionized water. Using HCl, adjust the pH to 7.8 and bring to a final volume of 250 mL.

1.4.2. Dissolve 21.4 g of sucrose (342.29 g/mol; final concentration = 0.25 mM) in 150 mL of deionized water.

1.4.3. Add 5 mL of Tris stock to sucrose to achieve a 10 mM final Tris concentration.

1.4.4. Add 0.5 mL of 0.5 M EDTA stock to Tris-sucrose to achieve a 1 mM final concentration.

1.4.5. Check the pH and adjust it to 7.4.

1.4.6. Bring to a final volume of 250 mL with deionized water and store at 4 °C.

1.5. Bovine erythrocyte Cu/Zn superoxide dismutase (SOD) stock (30,000 U/mL)

1.5.1. Reconstitute 30,000 U of SOD in 1 mL of PBS (approximately 5.7 mg, depending on activity of SOD lot).

1.5.2. Mix well, aliquot, and store at -20° C for short-term (6-12 months) and at -80 °C for long-term storage.

1.6. SOD working solution (1000 U/mL)

1.6.1. Transfer a 30 µL aliquot of 30,000 U/mL SOD stock into a 1.5 mL tube.

1.6.2. Dilute to 1000 U/mL by adding 870 µL of sterile PBS.

1.6.3. Keep the solution on ice, and use it fresh.

1.7. Phorbol 12-myristate 13-acetate (PMA) stock (5 mM)

1.7.1. Dissolve 1 mg of PMA (616.83 g/mol) in 325 µL of DMSO (final concentration = 5 mM).

1.7.2. Aliquot a 5 mM PMA solution and store it at -20 °C.

1.8. PMA working solution (125 µM)

1.8.1. Dilute a 10 µL aliquot of 5 mM PMA stock into 390 µL of sterile PBS.

1.8.2. Keep the solution on ice and use it fresh.

1.8.3. For a vehicle control for PMA, use 10 µL of DMSO in 390 µL of PBS.

1.9. Diphenyliodonium chloride (DIP) (2.5 mM)

1.9.1. Dissolve 3.2 mg of DIP (316.57 g/mol) in 4 mL to obtain a 2.5 mM stock.

1.9.2. Prepare the solution and use it fresh.

1.10. Deferoxamine mesylate salt (DFO) (20 mM)

1.10.1. Dissolve 4.5 mg of DFO (656.79 g/ mol) in 340 µL to obtain a 20 mM stock.

1.10.2. Prepare the solution and use it fresh.

1.11. Preparation of antimycin A (AA) stock (5 mM)

1.11.1. Dissolve 5.4 mg of AA (532 g/mol) in 2 mL of ethanol (final concentration = 5 mM).

1.11.2. Aliquot the stock in glass vials and store at -20 °C.

1.12. Preparation of spin probes

1.12.1. Bubble 50 mM phosphate buffer containing 100 µM DTPA with nitrogen for 30 min to remove dissolved oxygen from the buffer.

1.12.2. Remove the spin probe from the -20 °C freezer and allow the container to come to RT (10-15 min).

1.12.3. Weigh out 2.4 mg of 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine·HCl (CMH) (237.8 g/mol)

1.12.4. Dissolve CMH into 1 mL of the deoxygenated phosphate buffer for a final concentration of 10 mM.

1.12.5. Weigh out 5 mg of 1-hydroxy-4-[2-triphenylphosphonio)-acetamido]-2,2,6,6-tetramethylpiperidine,1-hydroxy-2,2,6,6-tetramethyl-4-[2-(triphenylphosphonio)acetamido]piperidinium dichloride (mito-TEMPO-H) (529.1 g/mol).

1.12.6. Dissolve mito-TEMPO-H into 1 mL of the deoxygenated phosphate buffer for a final concentration of 9.5 mM.

1.12.7. Weigh out 4.9 mg of 1-hydroxy-3-carboxy-2, 2, 5, 5-tetramethylpyrrolidine·HCl (CPH) (223.7 g/mol).

1.12.8. Dissolve CPH into 1 mL of the deoxygenated phosphate buffer for a final concentration of 22 mM.

1.12.9. Aliquot and store at -80 °C (freeze-thaw is not recommended).

2. Detection of Superoxide *in vitro*

2.1. Detection of total, extracellular, and intracellular superoxide in PMA-stimulated RAW 264.7 cells at RT

2.1.1. Following proper aseptic technique, thaw RAW 264.7 cells and passage them in DMEM media supplemented with 10% FBS (low endotoxin-free) and 1% antimycotic/ampicillin at 37 °C in CO_2 incubator.

2.1.2. Seed RAW 264.7 cells at 1 x 10^6 cells/well into 6-well plates one day prior to treatment.

2.1.3. Gently remove media and wash the cells once with 1 mL of KHB buffer.

2.1.4. Add KHB containing 100 µM DTPA to each well, and treat in a total volume of 500 µL with the following:

2.1.5. For wells pretreated with SOD, add 15 µL/well of SOD working solution (1000 U/mL; final concentration of SOD = 30 U/mL) and incubate for 10 min at 37 °C prior to addition of CMH and PMA.

2.1.6. Add 12.5 µL/well of 10 mM CMH stock (final concentration = 0.25 mM).

2.1.7. Add 40 µL/well of 125 µM PMA working solution (final concentration = 10 µM) or 40 µL vehicle (stock 10 µL of DMSO in 390 µL of PBS).

2.1.8. Incubate for 50 min at 37 °C in a CO_2 incubator.

2.1.9. Remove the plates from the incubator and place them immediately on ice.

2.1.10. Collect buffer from each well in separate, 1.5 mL, labeled tubes. Keep on ice throughout.

2.1.11. Add 100 µL of fresh KHB buffer containing 100 µM DTPA, gently scrape the cells, and resuspend by pipetting up and down several times. Keep on ice throughout cell resuspension.

2.1.12. Load the sample collected in steps 2.1.10 and 2.1.11 (50 µL) in each of the capillary tubes. Seal both ends and run the EPR.

NOTE: Always test a tube or well (without cells) containing the probe in buffer (same concentration = 0.25 mM), treated under the same conditions as the cells (same incubation time and temperature) as a control, since background intensity of the probe is temperature- and time-dependent.

2.1.13. Set the EPR acquisition parameters to the following: microwave frequency = 9.65 GHz; center field = 3432 G; modulation amplitude = 2.0 G; sweep width = 80 G; microwave power = 19.9 mW; total number of scans = 10; sweep time = 12.11 s; and time constant = 20.48 ms.

2.2. Detection of mitochondrial superoxide in RAW 264.7 cells

2.2.1. Follow steps 2.1.1 and 2.1.2 to seed RAW 264.7 cells one day prior to the experiment.

2.2.2. Remove media and wash the cells once with 1 mL of KHB buffer.

2.2.3. Add 200 µL of KHB containing 100 µM DTPA to each well.

2.2.4. Add 5.3 µL/well of 9.5 mM mito-TEMPO-H stock (final concentration = 0.25 mM)

2.2.5. Incubate for 10 min at RT.

2.2.6. Add 1 µL/well of antimycin A (AA), 5 mM stock solution in ethanol (final concentration = 25 µM).

2.2.7. Incubate for 50 min at 37 °C in a CO_2 incubator.

2.2.8. Remove the plates from the incubator and place them immediately on ice.

2.2.9. Gently scrape the cells and resuspend by pipetting up and down. Keep on ice.

2.2.10. Load the sample in a capillary tube. Seal both ends.

2.2.11. See the previous section for EPR setting.

2.3. Detection of superoxide in RAW 264.7 cells at 77 K

2.3.1. Place the buffer collected in step 1.1.10 in pre-prepared PTFE tubing 1-2 inches in length (3/16" OD x 1/8" ID). Make sure the PTFE tubing is straight so it can be easily inserted and removed from the finger dewar. Use a rubber stopper to close one end of the PTFE tubing, pipette the buffer or cell suspension (100 to 150 µL) into the PTFE tubing, and seal the tubing with a second stopper.

2.3.2. Flash freeze the sample in liquid nitrogen. The sample can be transferred to a labeled cryopreservation tube for storage at -80 °C or run immediately.

2.3.3. Fill the finger dewar with liquid nitrogen and insert the PTFE tubing containing the sample into the finger dewar. Make sure the sample is centered in the active space of the resonator and run EPR at 77 K.

NOTE: Start the nitrogen gas flow to your spectrometer 15-30 min before the measurements, and continue this flow throughout the measurements to prevent water condensation in the resonator.

2.3.4. Set EPR acquisition parameters to the following: microwave frequency = 9.65 GHz; center field = 3438 G; modulation amplitude = 4.0 G; sweep width = 150 G; microwave power = 0.316 mW; total number of scans = 10; sweep time = 60 s; and time constant = 1.28 ms.

3. EPR Measurements in Fluids

3.1 Whole blood

3.1.1 Treat mice (8-12 weeks old) with a single dose of intratracheal bleomycin (Bleo; 100 µL at 1 U/mL) dissolved in PBS or PBS alone as previously described[16-17].

3.1.2 Euthanize mice by administering inhaled isoflurane (1.5-4%) followed by exsanguination and cervical dislocation. Aspirate blood through the right ventricle into a syringe coated with heparin (1000 USP/mL) containing 100 µM DTPA and transfer to a 1.5 mL tube.

3.1.3 In a separate 1.5 mL tube, add 15 µL of PBS containing 100 µM DTPA and 3 µL of CMH (10 mM) to 132 µL of blood for a total volume of 15 µL and final CMH concentration of 0.2 mM.

3.1.4 Incubate blood for 10 min at 37 °C in a water bath.

3.1.5 Remove the tubes from water bath.

3.1.6 Take an aliquot by loading blood in a capillary tube and run EPR at RT with the following EPR acquisition parameters: microwave frequency = 9.65 GHz; center field = 3432 G; modulation

amplitude = 1.0 G; sweep width = 80 G; microwave power = 19.9 mW; total number of scans = 3; sweep time = 12.11 s; and time constant = 20.48 ms. Alternatively, samples can be flash frozen as described in step 2.3 for measurements at 77 K. EPR acquisition parameters are the following: microwave frequency = 9.65 GHz; center field = 3438 G; modulation amplitude = 4.0 G; sweep width = 150 G; microwave power = 0.316 mW; total number of scans = 2; sweep time = 60 s; and time constant = 1.28 ms.

3.2 Bronchoalveolar lavage fluid (BALF)

3.2.1 After euthanasia (see step 3.1.2), collect BALF by slowly instilling and withdrawing 1 mL of PBS containing 100 µM DTPA three times in a syringe *via* a cannula placed in the trachea.

3.2.2 In a 1.5 mL tube, treat 200 µL of BALF with 4 µL of CMH (10 mM) to obtain a final concentration of 0.2 mM.

3.2.3 Incubate BALF for 50 min at 37 °C in a water bath.

3.2.4 Take tubes out of the water bath and place them on ice.

3.2.5 Load BALF in a capillary tube and run EPR at RT with the same EPR settings as used in step 1.1.13, or flash freeze in liquid nitrogen as described in step 2.3.

3.3 EPR measurements in blood and BALF at 77 K

3.3.1 Follow the protocol above to collect blood (steps 3.1.1. to 3.1.4) and BALF (steps 3.2.1 to 3.2.4).

3.3.2 Place 150 µL of the treated blood or BALF in PTFE tubing (1-2 in). Use a rubber stopper to close one end of the PTFE tubing prior to adding the sample and another stopper to seal the tubing.

3.3.3 Flash freeze the sample in liquid nitrogen.

3.3.4 See section 2.3 for details on running EPR in frozen samples in PTFE tubing using the finger dewar at 77 K.

4 EPR Measurements on Lung Tissue

4.1 Flash frozen lung tissue

4.1.1 After collecting the BALF in step 3.2.1, the chest is opened and lungs flushed with 10 mL of cold PBS *via* the right ventricle to remove blood. Flash freeze the lung tissue in liquid nitrogen. Frozen lung tissue can be stored at -80 °C until use for EPR measurements.

4.1.2 Stabilize the lung tissue on dry ice with tweezers and cut multiple small pieces (5-15 mg) of lung tissue using a single-edge blade.

4.1.3 Weigh the tissue in a 1.5 mL tube, place the tube on the scale and tare the scale, then add the tissue pieces and record the weight.

4.1.4 To the tissue in the 1.5 mL tube, add 196 µL of KHB containing DTPA and 4 µL of CMH (0.2 mM) to achieve a 200 µL total volume.

4.1.5 Incubate for 1 h at 37 °C in a water bath.

4.1.6 Spin down (for a few seconds) in a microcentrifuge at 3,884 x g.

4.1.7 Place on ice and pipette 150 µL of the supernatant into the PTFE tubing and freeze for the 77 K measurements as described in section 2.3.

NOTE: For this method, the heterogeneity of the injury needs to be considered. For a bleomycin-induced lung injury, given that it is a highly heterogeneous injury, it is recommended to cut several tissue pieces from different parts of the lung from each mouse. Alternatively, a larger piece of tissue can be homogenized in KHB buffer containing 100 µM DTPA at a 1:6 weight-to-volume ratio (mg/µL) as described below.

4.2 Fresh lung tissue preserved in sucrose buffer

4.2.1 Flush the lavaged lungs with cold PBS to remove blood as done in step 3.1.2.

4.2.2 Homogenize the fresh lung tissue in Tris-EDTA buffer containing 0.25 M sucrose with a 1:6 lung/buffer (mg/µL) ratio using Dounce tissue grinder with a glass or PTFE pestle.

4.2.3 Add 50 µL of the lung homogenate to 450 µL of KHB containing 100 µM DTPA.

4.2.4 In a 1.5 mL tube (in a total volume of 100 µL), to 98 µL of lung homogenate in KHB, add 2 µL of CMH of 10 mM stock to obtain a final concentration of 0.2 mM.

4.2.5 Incubate for 20 min 37 °C in a water bath.

4.2.6 Place the samples on ice and load them in a capillary tube. Run EPR at RT (settings used in step 2.1.13).

4.2.7 To test the contribution of specific species and sources using different inhibitors, pre-treat 88 µL of lung homogenate +/- inhibitor, adjusting with KHB to achieve a final volume of 98 µL. In this experiment, the inhibitors included 10 µL of SOD (100 U/mL), 4 µL of deferoxamine (DFO; final concentration = 800 µM), or 4 µL of diphenyliodonium chloride (DIP; final concentration = 100 µM). Incubate for 20 min at 37 °C in a water bath.

4.2.8 Add 2 µL of CMH and incubate for another 20 min at 37 °C, followed by EPR measurements as described above. Include a one-time matched blank sample with CMH KHB containing sucrose buffer. Alternatively, store aliquots of the remaining lung homogenates (step 3.1.2) at -80 °C for future measurements.

NOTE: The total volume can be scaled as needed.

4.3 **EPR measurements on lung tissue from mice injected with spin probes *in vivo* (at RT using tissue cell)**

4.3.1 Prepare CPH stock solution by dissolving 4.9 mg of CPH in 1 mL of filtered and deoxygenated 50 mM phosphate buffer.

4.3.2 Anesthetize mice with inhaled isoflurane (1.5-4%) for 20-30 seconds until unresponsive to toe pinch. Inject mice *via* retroorbital route with 100 µL of CPH spin probe for a 25 g mouse body weight (final dose = 20 mg/kg), and allow the probe to circulate for 1 h. Immediately after retroorbital injection, add one drop of 0.5% proparacaine HCl on the eye area to prevent eye pain and dryness. Monitor mice for 1 h and proceed to tissue harvesting.

4.3.3 Harvest the lung tissue as described above and flash freeze the lungs.

4.3.4 Cut 20-30 mg of frozen tissue on dry ice and record the exact weight.

4.3.5 Gently wipe the tissue with cleaning wipes to absorb any surface water.

4.3.6 Place the tissue within the window of the tissue cell (an accessory allows EPR measurements for tissue samples) and run EPR to determine total spins. The data can be expressed as total spins per mg of tissue.

5 Data Analysis

5.1 Simulate the EPR spectra using SpinFit module incorporated in the Xenon software of the bench-top EMXnano EPR spectrometer. Determine the nitroxide concentration by the SpinCount module. Alternatively, a calibration curve of a stable nitroxide such as 4-hydroxy-TEMPO or TEMPOL can be made, and the concentration can be obtained by comparing the intensity of the signal with the sample and standard.

5.2 For the data collected at 77 K, use double integration followed by SpinCount.

REPRESENTATIVE RESULTS:

Superoxide detection using CMH was validated using the X/XO superoxide generating system to demonstrate that the nitroxide ($CM^{\cdot}$) signal was fully inhibited by SOD, while catalase had no effect. (**Figure 1A**) The total, extracellular superoxide was then evaluated in RAW 264.7 cells by

incubating cells with the cell-permeable CMH spin probe +/- SOD pretreatment. The nitroxide concentration was measured in both the cell suspension and buffer, which demonstrated that the values in the two sample types were similar due to the permeable nature and rapid equilibration of the spin probe. (**Figure 1B**) The nitroxide radical signal increased in RAW 264.7 cells stimulated with PMA compared to control cells. This signal was significantly attenuated in cells pretreated with cell-impermeable SOD. Each color represents wells tested on different days, demonstrating the consistency of data collected on specific days and reproducibility of the results across time. The concentration of extracellular superoxide was determined by subtracting the signal in PMA cells pretreated with SOD from the signal after PMA in the absence of SOD (T). The remaining signal was attributed to intracellular superoxide (**Figure 1C**). **Figure 1D** illustrates the calculation of total and extracellular superoxide. (E) The intracellular signal was confirmed in PMA-treated cells after removal of the media and by the effect of PEG-SOD on the signal. In this graph, in contrast to (C), the CMH blank was not subtracted from the measurements, and the raw data is shown.

Mitochondrial superoxide in RAW 264.7 cells was detected using the EPR spin probe mito-TEMPO-H, which accumulates in mitochondia. (A) Representative EPR spectra for the baseline mito-TEMPO-H signal in buffer, the increased mito-TEMPO-H signal in control cells (Con), and the further enhanced signal in cells stimulated with the mitochondrial inhibitor Antimycin A (AA). The increase in the signal was attributed to the mitochondrial superoxide based on our previous study showing that SOD2 overexpression significantly attenuated measurements with mito-TEMPO-H[10]. In **Figure 2B**, the mitochondrial nitroxide concentration was determined by subtracting the mito-TEMPO-H signal in time-matched buffer from the cell measurements. The $CM^{\cdot}$ signal obtained at low temperatures in RAW 264.7 cells after stimulation with PMA in the presence and absence of SOD. (**Figure 3A**) The $CM^{\cdot}$ signal was attenuated in the presence of SOD, consistent with the room temperature data (**Figure 1**). **Figure 3B** shows the photograph of PTFE tubing with the stoppers used to collect data at 77 K for cells and *in vivo* samples. Superoxide production was detected in blood and BALF using the CMH spin probe. Blood or BALF samples were collected from PBS- and Bleo-treated mice and incubated immediately with CMH. The samples were transferred to the PTFE tubing and flash frozen, and EPR data was collected at 77 K. The concentration of nitroxide ($CM^{\cdot}$) accumulated in blood incubated with CMH (0.2 mM) at 37 degrees for 10 min. (**Figure 4A**). Nitroxide ($CM^{\cdot}$) concentration from BALF incubated for 50 min. (C) Nitroxide concentration represents the concentration of ($CM^{\cdot}$) accumulated in volume of blood or BALF used in the experiment (**Figure 4B**).

Three methods have been tested to evaluate several published techniques for tissue preservation and administration of spin probes *ex vivo vs*. *in vivo.* To perform EPR measurements on lung tissue, we first used flash frozen lung tissue from control or injured mice. **Figure 5A** shows the total $CM^{\cdot}$ signal in the supernatant of a small piece of lung tissue incubated at 37 °C with CMH in PBS- and Bleo-treated mice, respectively. Due to heterogeneity of the lung injury after Bleo treatment, it is recommended to cut pieces from different regions of the lung and average several measurements to provide a more representative value. Alternatively, one can homogenize the entire lung and use one sample of this homogenate. Data collected at 77 K using PTFE tubing and

finger dewar. **Figure 5B** shows representative spectra of nitroxide ($CM^{\cdot}$) signals from PBS- and Bleo-treated mice, respectively.

One limitation to treating lung tissue *ex vivo* is that it is not possible to reliably distinguish extracellular from intracellular superoxide due to the processing of the tissue that disrupts cell membranes. If this information is important to the experimental question, it can be addressed by using the *in vivo* CPH instillation method described below. Frozen tissue cannot be used to assess mitochondrial superoxide; though, for this measurement, the protocol can be adapted to use mito-TEMPO-H in the tissue or freshly isolated mitochondria.

As a second method for EPR measurements in lung tissue, fresh tissue was homogenized in sucrose buffer. The lung homogenate was incubated with CMH probe in KHB buffer containing DTPA. EPR measurements were carried out at RT. **Figure 6A** demonstrates the increase in $CM^{\cdot}$ with Bleo. We presented an additional testing using different inhibitors that can be used to determine the species that contribute to the $CM^{\cdot}$ signal. To elucidate the origin of $CM^{\cdot}$ signal generated from lung tissue, we pretreated the lung homogenates with several scavengers and enzymes inhibitors. Lung homogenates were incubated with CMH in the absence or the presence of SOD, deferoxamine (DFO), and diphenyliodonium chloride (DIP) to account (respectively) for the contributions from superoxide, iron, or superoxide generated from flavin-containing enzymes (**Figure 6B**). This approach can be adapted to assess the specific radical species generated in a system or elucidate the contribution of other enzymatic sources (*e.g.*, NOX, eNOS, or xanthine oxidase).

Mice were injected with CPH spin probes (20 mg/kg) *via* the retroorbital route to perform EPR measurements *in vivo*. It is unknown whether CMH can be safely administered to animals, while the CPH probe has been reported to be non-toxic; thus, we selected CPH for the *in vivo* experiments. Lung tissues were harvested and flash frozen in liquid nitrogen 1 h after circulation of CPH probes. Mice can be simultaneously treated with specific antioxidants to differentiate the species responsible for the signal. **Figure 7A** shows the higher $CP^{\cdot}$ signal in Bleo-treated mice compared to control mice. Representative spectra of lung tissue from control and Bleo-treated mice are shown in **Figure 7B**. A mixed EPR spectra of $CP^{\cdot}$ and ascorbic acid radical was observed. The values reported in **Figure 7A** are the concentrations of $CP^{\cdot}$ components. Data were collected at RT using the tissue cell.

FIGURE LEGENDS:

Figure 1: Detection of superoxide in different cell compartments. (A) EPR spectra generated by 0.25 mM CMH in 0.5 mM hypoxanthine/xanthine oxidase (8 mU/mL) with and without SOD (30 U/mL). (B) RAW 264.7 cells (1 x 10^6 cells/well) were stimulated with 10 µM PMA in the presence of CMH for 50 min at 37 °C and nitroxide concentration (µM) detected in cell suspension (cells + buffer) and buffer collected from treated cells. (C) RAW 264.7 cells were stimulated with PMA *vs*. vehicle control (Con). One set of cells were pretreated for 10 min with 30 U/mL cell-impermeable SOD (PMA + SOD). Each color represents data from different experimental days and each point represents cells from an individual well. The nitroxide signal in a time-matched blank with CMH in KHB was subtracted from each signal to obtain final values. (D) Calculation of total and

extracellular superoxide in PMA stimulated cells; T = total superoxide, EC = extracellular superoxide (SOD inhibitable signal). (E) To evaluate the intracellular superoxide signal (IC), the signal in buffer after PMA + SOD was compared to PMA-treated cells after the removal of buffer. To confirm, wells were pretreated with 60 U/mL cell-permeable PEG-SOD for 1.5 hours to determine the intracellular SOD inhibitable. The time-matched CMH blank is shown, and data reflect absolute nitroxide signal. Data expressed as mean ± SEM.

Figure 2: Detection of mitochondrial superoxide in RAW cells stimulated with antimycin A. (A) Representative spectra of the mitochondrial-specific EPR spin probe, 0.25 mM mito-TEMPO-H in RAW 264.7 cells without (Con) or with 25 µM antimycin A (AA) for 50 min at 37 °C. (B) $CM^{\cdot}$ concentration (µM) in cells treated with AA compared to control. The nitroxide signal in a time-matched mito-TEMPO-H blank was subtracted from total signal to obtain final values. Data expressed as mean ± SEM.

Figure 3: Detection of superoxide in RAW 264.7 cells at 77K. (A) RAW 264.7 cells stimulated with 10 µM PMA and EPR spin probe, CMH 0.25 mM (50 min at 37 °C) with (black) or without (red) pretreatment with 30 U/mL SOD. 100 µL of supernatant was loaded in a 1-inch in length piece of PTFE tubing, then flash frozen in liquid nitrogen. The stoppers were removed, and frozen PTFE tubing was placed in the finger dewar for data acquisition at 77 K. (B) A photo of PTFE tubing and stoppers.

Figure 4: EPR measurements in blood and BALF from control and bleomycin-treated mice. Mice were treated with a single dose of intratracheal bleomycin (IT Bleo) (100 µL at 1 U/mL) or PBS vehicle. At 7 days, mice were anesthetized and euthanized. Blood was collected *via* right ventricular puncture into a syringe coated with 1000 USP/mL heparin containing 100 µM DTPA. Bronchoalveolar lavage fluid (BALF) was collected by lavaging the lungs with 1 mL of 100 µM DTPA in PBS. Blood and BALF were incubated for 10 or 50 min, respectively, with 0.2 mM CMH at 37 °C. 150 µL of blood or BALF was loaded in PTFE tubing flash frozen in liquid nitrogen and EPR data collected at 77 K using a finger dewar. Data show nitroxide concentrations in (A) blood and (B) BALF from PBS- and Bleo-treated mice (n = 4-6). Data expressed as mean ± SEM. (C) Representative spectra of nitroxide in blood from PBS- and Bleo-treated mice.

Figure 5: EPR measurements in flash frozen lung tissue. Mice were treated with a single dose of intratracheal bleomycin (IT bleo) (100 µL at 1 U/mL) or PBS vehicle. At 7 days, the lungs were flushed with cold PBS to remove blood and flash frozen in liquid nitrogen. 5-15 mg of flash-frozen lung tissue was incubated with 0.2 mM CMH in KHB containing 100 µM in 200 µL of total volume for 1 h at 37° C. Supernatant was collected and placed in PTFE tubing and run at 77 K in the finger dewar. (A) Nitroxide concentration (µM of nitroxide normalized to 1 mg of tissue). Data represent the average of 2-3 measurements for each lung. Data expressed as mean ± SEM. (B) Representative spectra of nitroxide in lung tissue from PBS- and Bleo-treated mice.

Figure 6: EPR measurements in lung tissue preserved in sucrose buffer. Mice were treated with a single dose of intratracheal bleomycin (100 µL at 1 U/mL). At 7 days post-treatment, the lungs were flushed with cold PBS to remove blood, and fresh lung tissue was homogenized in Tris-EDTA

buffer containing 0.25 mM sucrose at a 1:6 lung weight/buffer volume (mg/µL) ratio. 50 µL of lung homogenate was preincubated with KHB with or without the following inhibitors for 20 min at 37 °C: SOD (100 U/mL), deferoxamine (DFO; 800 µM), and diphenyliodonium chloride (DIP); followed by incubation with 0.2 mM CMH in KHB containing 100 µM DTPA for 20 min at 37 °C. Data was obtained at RT using EPR capillary tubes. (A) Nitroxide concentration in lungs from PBS- and Bleo-treated mice. (B) Nitroxide concentration in Bleo lungs in the absence or the presence of inhibitors (n=3). Data expressed as mean ± SEM.

Figure 7: EPR measurements in lung tissue from mice injected with CPH spin probe. 100 µL of CPH was administered *via* retroorbital injection for a final concentration of 20 mg of CPH per kg of body weight. After 1 h of circulation, mice were euthanized, lungs were flushed with 10 mL of cold PBS *via* the right ventricle, and lung tissue was flash frozen. 20 to 30 mg of lung tissue was placed in tissue cell and EPR measurements performed at RT. (A) Data expressed as spins/mg. (B) Representative spectra of nitroxide signal in PBS and Bleo lung tissues (* indicates the overlap with ascorbic acid radical). Data expressed as mean ± SEM.

Table 1. Common inhibitors used to distinguish species responsible for spin probe oxidation.

DISCUSSION:

The assessment of free radical production in biologicalsettings is important in understanding redox regulated signaling in health and disease, but the measure of these species is highly challenging due to the short half-life of free radical species and technical limitations with commonly used methods. EPR is a valuable and powerful tool in redox biology, as it is the only unambiguous method for detecting free radicals. In this project, we demonstrate practical EPR methods for designing experiments and preparing samples to detect ROS using spin probes in different cellular compartments *in vitro* and different tissue compartments *in vivo.* We also provide practical methods to handle biologicalsamples and store samples to improve efficiency.

Spin probes react efficiently with ROS and produce a stable nitroxide radical that can be detected with EPR. Several derivatives of the spin probe (cyclic hydroxylamine) have been synthesized with different permeability characteristics, which make them suitable for detecting free radical production in different cellular compartments[10]. This protocol utilized the cell-permeable spin probe, CMH; though, the impermeable spin probe 1-hydroxy-2,2,6,6-tetramethylpiperidin-4-yl-trimethylammonium chloride HCl (CAT1H) can be used to detect extracellular superoxide. Similar to our prior study in human lymphoblast cell lines[18], we were able to validate use of the permeable CMH spin probe with impermeable SOD and cell permeable PEG-SOD in RAW264.7 cells (a mouse lung macrophage cell line) stimulated with PMA to differentiate between extracellular and intracellular superoxide.

We also validated the rapid equilibration of CMH between the intra- and extra-cellular compartments, and we also found that the superoxide signal in cells drops significantly after washing the cells only once with KHB (data not shown). We confirmed utility of the mitochondrial specific spin probe mito-TEMPO-H in RAW 264.7 cells to measure the increased mitochondrial superoxide generated upon stimulation with mitochondrial electron transport chain inhibitor

antimycin A. The specific contribution of mitochondrial superoxide production to the mito-TEMPO-H has been previously demonstrated and can be validated in experiments using isolated fresh mitochondria or systems with mitochondrial superoxide dismutase MnSOD (SOD2) overexpression[10].

The assessment of ROS production *in vivo* is particularly challenging, but the ability to detect production of specific ROS provides important information when interrogating the role of oxidative stress or redox regulated signaling in biological settings. The appropriate handling of tissue when using spin probes and EPR is essential to generate reproducible and meaningful results. The use of spin probes with tissue will not likely measure superoxide radicals present at the time of tissue harvesting due to a short half-life, but instead it detects superoxide produced by enzymes such as NAPDH oxidase, uncoupled endothelial nitric oxide synthase, or xanthine oxidase when lung tissue or homogenates are incubated with the spin probe at 37 °C. The use of frozen tissue will not include superoxide generated by mitochondria, since freezing damages mitochondrial electron transport chain activity. To test mitochondrial superoxide, investigators need to isolate fresh mitochondria or use mitochondrial specific probes *in vivo* or in fresh tissue.

Several different protocols to preserve tissue have been published in the literature[14,15]. We compared three published methods for EPR measurements in lung tissue: 1) flash freezing tissue in liquid nitrogen, 2) homogenizing tissue in sucrose buffer, and 3) treating mice *in vivo* with a spin probe 1 hour before tissue harvesting. We compared control mice to mice with severe lung inflammation and oxidative stress induced by bleomycin to test each method's ability to show consistent differences in nitroxide signals in injured lungs. All three methods showed a similar relative increase in nitroxide signal in the lungs of bleomycin-treated mice. The use of flash frozen tissue would likely be the easiest approach to collect tissue for most labs, negating the need to process tissue in the sucrose buffer at the time of harvesting. The injection of CPH to capture free radicals *in vivo* is powerful, but to confirm the specific species, this requires a treatment group including the appropriate antioxidant.

One challenge of using spin probes is that the oxidation of spin probes to nitroxide generates a similar three-line EPR spectrum regardless of the species responsible for the oxidation; thus, it does not distinguish between different ROS species. Also, it has been reported that there are potential reactions of hydroxylamine probes with photosynthetic electron transport chain and cytochrome c oxidase[19,20]. These observations should be considered when interpreting results. In this protocol, the photosynthetic system is not present, and the inclusion of DTPA with the buffer inhibits potential contamination of free ferric and cuprous ions[10] . We demonstrated how to use a series of specific enzymes or chelators in lung tissue to establish the contribution of particular ROS or enzyme inhibitors to determine the source of ROS. This approach has been previously used with EPR to determine the contribution of ROS due to uncoupled eNOS[13,15]. We provide a list of common inhibitors used to distinguish species responsible for spin probe oxidation (**Table 1**).

We also demonstrated the importance of optimizing the incubation time for each experimental condition. When comparing spin probes to spin traps, spin traps generate unique spectra

depending on the reactant which allows for specificity of the free radical species; however, they also exhibit slow kinetics for superoxide spin trapping and are prone to biodegradation. The treatment of lung tissue with the EPR probe *ex vivo* is also limited by an inability to adequately distinguish extracellular from intracellular superoxide due to the disruption of cell membranes during processing of the tissue (freezing or homogenizing). Use of the injected spin probe *in vivo* in conjunction with SOD or cell-permeable PEG-SOD can address this problem.

One goal was to establish a protocol to efficiently collect samples and store them at -80 °C prior to EPR measurements. We therefore developed a practical method to use PTFE tubing for holding the samples. This tubing is placed directly into the finger dewar for EPR analysis at 77 K without the need to clean the dewar between samples. This is an alternative to the recently published method involving the freezing of samples in 1 mL syringes. The measurements in frozen samples stored in PTFE tubing can be repeated over several days to demonstrate stability of the signal. This approach allows for batching the EPR measurements and facilitates transferring of the samples between laboratories so a remote EPR facility can run samples.

Overall, these protocols provide a straightforward approach to preparing cells and tissues for EPR measurements in biological systems. The protocols can be adapted to other models associated with oxidative stress and with the use of other spin probes. The timing and concentration of the spin probe will need to be adjusted for each experimental condition. The ability of EPR to determine the presence and production of free radical species unambiguously provides rigor to experimental approaches in the field of redox biology.

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DISCLOSURES:

The authors have nothing to disclose.

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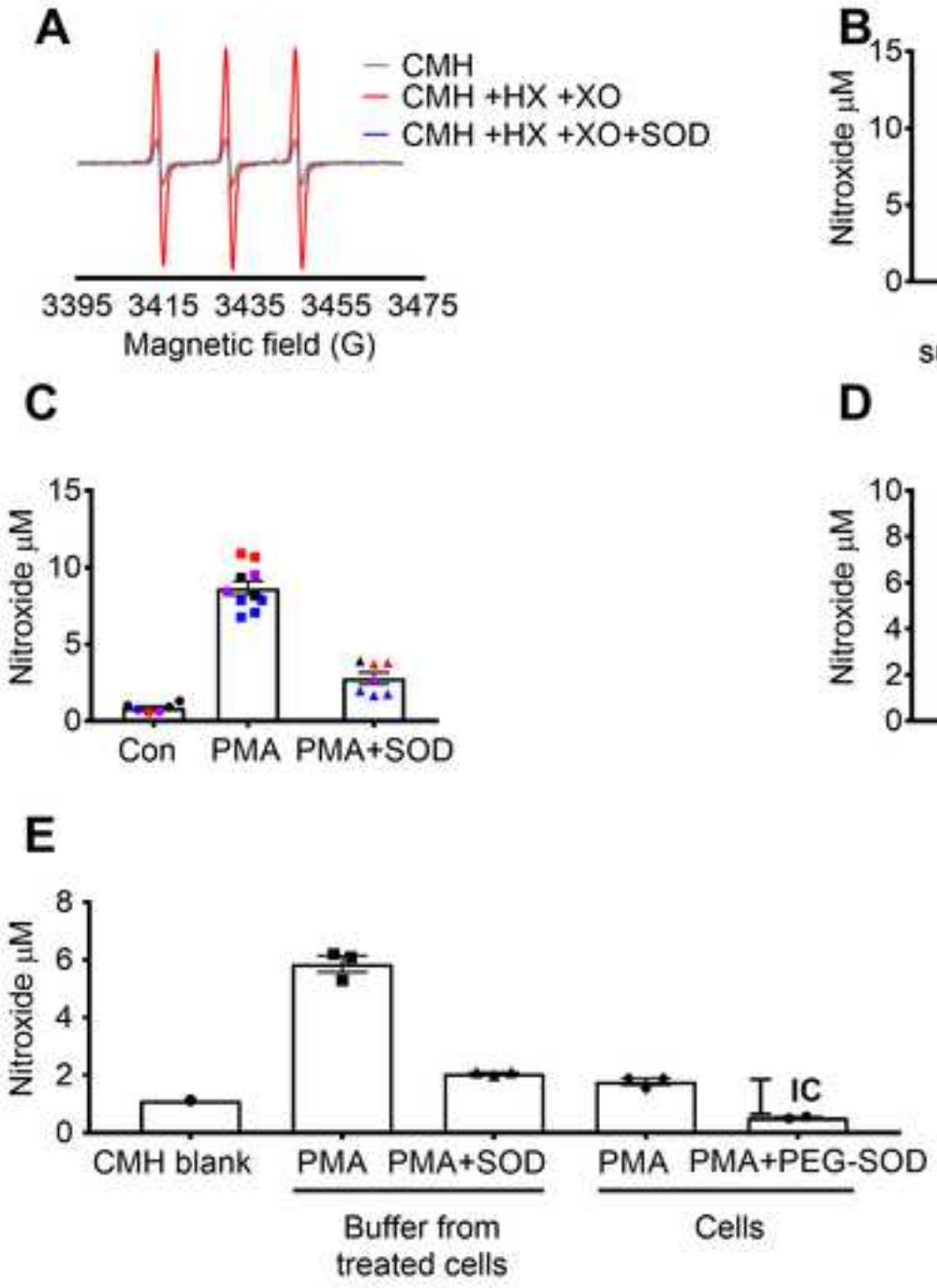
Figure



Figure

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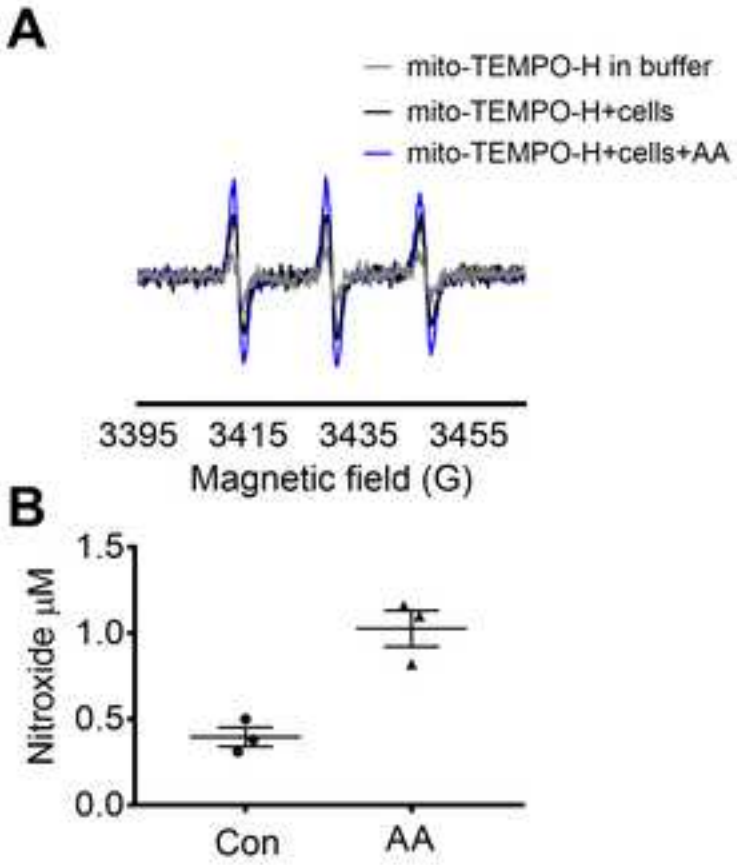

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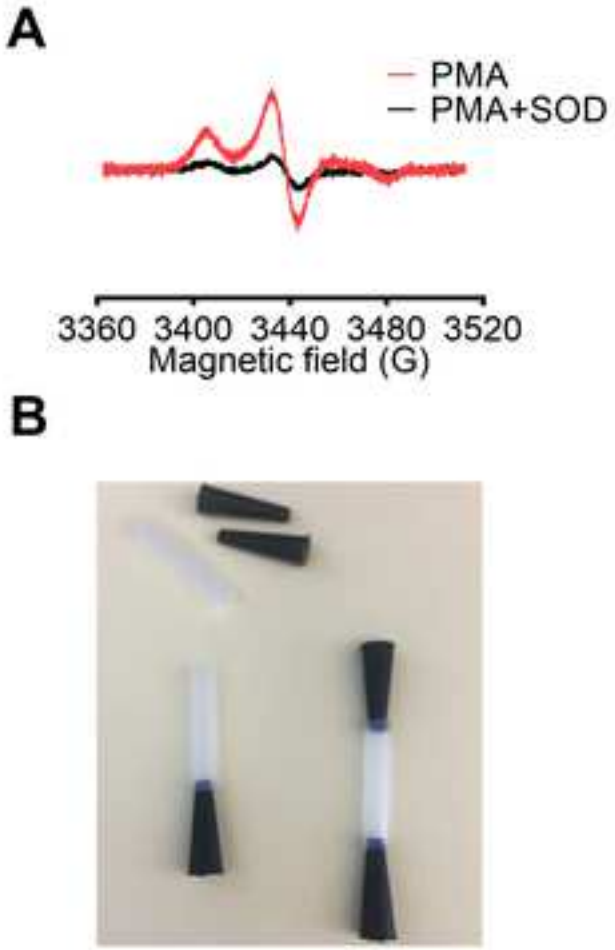

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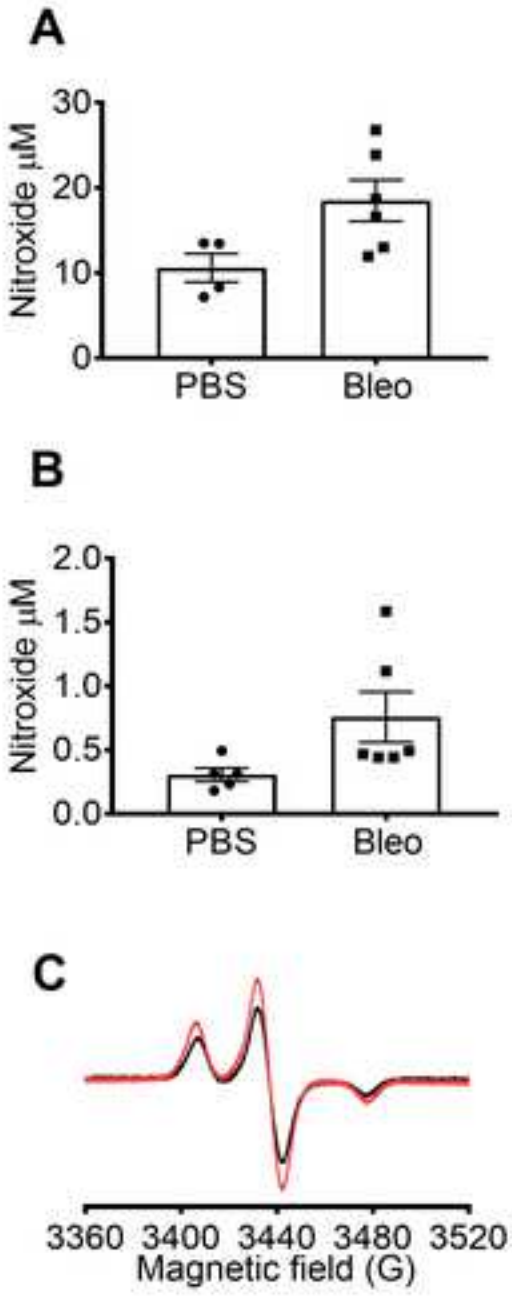

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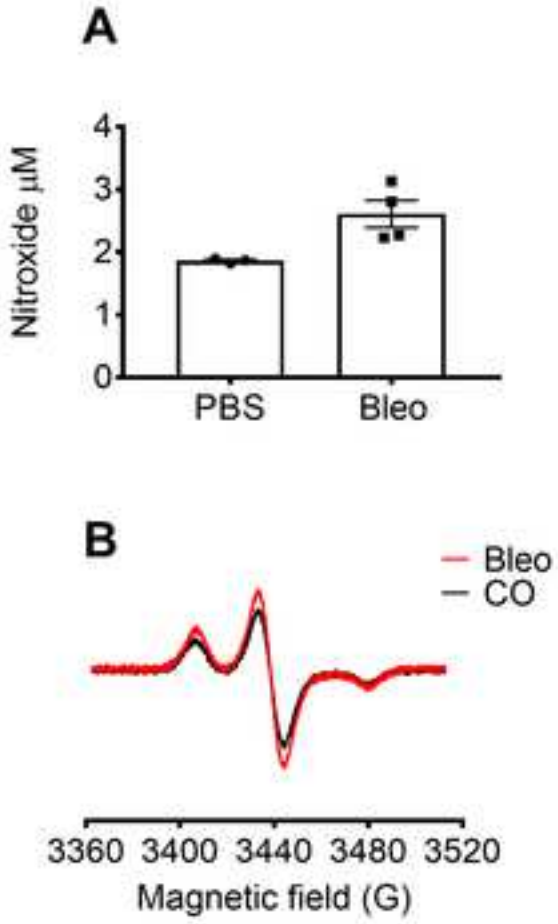

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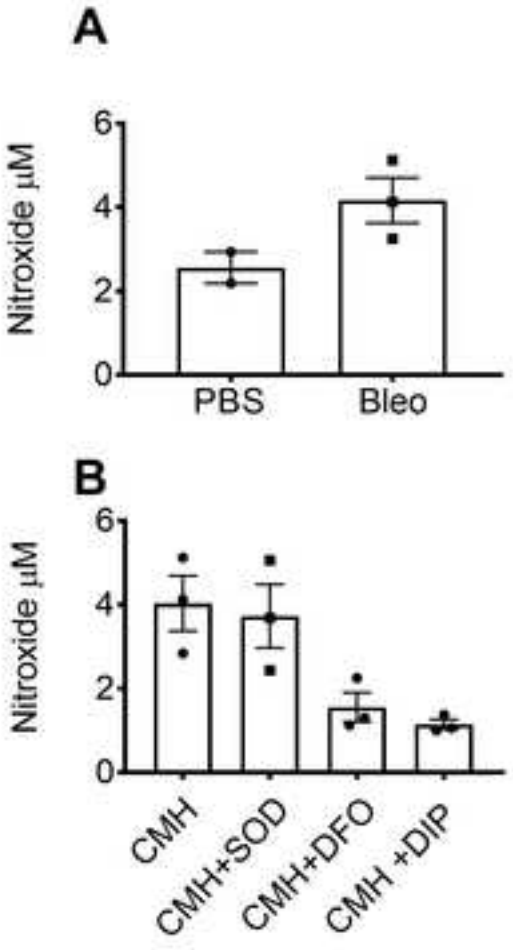

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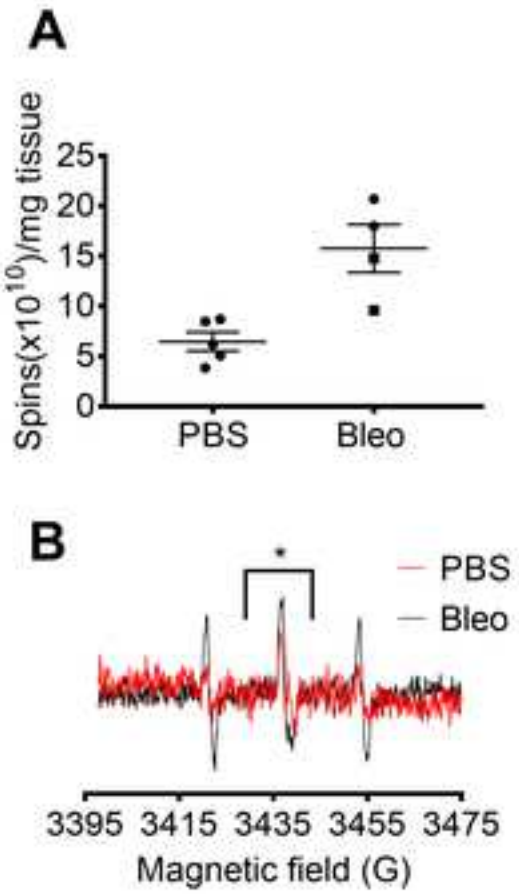

Table

Inhibitors	Species
Superoxide dismutase (SOD)	Extracellular superoxide
Superoxide dismutase–polyethylene glycol (PEG-SOD)	Intracellular superoxide
Catalase	Hydrogen peroxide based radicals
Urate	Peroxynitrate
Ethanol and DMSO	Hydroxyl radical
Metal chelators	Metal ions (iron and copper)

Name
DMEM
Diethylenetriaminepentaacetic acid (DTPA)
sodium chloride (NaCl)
potassium phosphate dibasic (HK_2PO_4)
potassium phosphate monobasic (KH_2PO_4)
Krebs-Henseleit buffer (KHB)
Bovine erythrocyte superoxide dismutase (SOD)
Phorbol 12-myristate 13-acetate (PMA)
Antimycin A (AA)
1-Hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine . HCl (CMH)
1-Hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine . HCl (CPH)
1-Hydroxy-4-[2-triphenylphosphonio)-acetamido]-2,2,6,6-tetramethylpiperidine, 1-Hydroxy-2,2,6,6-tetramethyl-4-[2-(triphenylphosphonio)acetamido]piperidinium dichloride (mito-TEMPO-H)
1-Hydroxy-2,2,6,6-tetramethylpiperidin-4-yl-trimethylammonium chloride . HCl (CAT1H)
Heparin
Diphenyliodonium chloride
Deferoxamin mesylate salt
Critoseal
BRAND disposable BLAUBRAND micropipettes, intraMark
PTFE FRACTIONAL FLUOROPOLYMER TUBING 3/16” OD x 1/8” ID
BER STOPPERS FOR NMR SAMPLE TUBES FOR THIN WALL TUBES HAVING AN OD OF 4mm-5mm (3.2mm TO 4.2mm
EMXnano Bench-Top EPR spectrometer
EMX NANO TISSUE CELL

Company	Catalog Number
LifeTech	10566-016
Sigma Aldrich	D6518-5G
Fisher Scientific	BP358-212
Fisher Scientific	BP363-500
Sigma Aldrich	P-5379
(Alfa Aesar, Hill)	J67820
Sigma Aldrich	S7571-30KU
Sigma Aldrich	P1585-1MG
Sigma Aldrich	A8674-25MG
Enzo Life Sciences	ALX-430-117-M050
Enzo Life Sciences	ALX-430-078-M250
Enzo Life Sciences	ALX-430-171-M005
Enzo Life Sciences	ALX-430-131-M250
Sagent Pharmaceuticals	NDC 25021-400-10
Sigma Aldrich	43088
Sigma Aldrich	D9533-1G
Leica	39215003
Sigma Aldrich	708733
NORELL	1598774A
NORELL	94987
Bruker BioSpin GmbH	E7004002
Bruker BioSpin GmbH	E7004542

Comments
cell culture media
used to prepare 50 mM phosphate saline buffer according to Sigma aldrish
used to prepare 50 mM phosphate saline buffer according to Sigma aldrish
used to prepare 50 mM phosphate saline buffer according to Sigma aldrish
Dissolve in DMSO
DISSolve in Ethanol and store in glass vials

Capillaries
Teflon tubing

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Article Title: Superoxide Detection in in vitro and in vivo Models Using Spin Probes

Signature: Eva Nozik-Grayck Date: 5/11/2018

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Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Thank you – we have carefully proofread the manuscript to correct spelling and grammar.

2. Figure 2: Please define the "+"/"-" symbols in the figure legend.

+ Sign represents with the treatment and – sign represents without the treatment
Figure 2 was deleted in the revised version. We think it is enough to state the observation without the figure.

3. Figure 3: Please line up panels A and B.

Panel B was removed since the typical CM· spectra at room temperature was shown already in figure 1

4. Figures 4, 6 and 7: Please define error bars in the figure legend.

Bars in the figures have been defined as standard error in the legend.

5. Title: Please revise the title to be less wordy.

Title is revised to "**Use of Electron Paramagnetic Resonance (EPR) in Biological Samples at Ambient Temperature and 77 K"**

6. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

We have edited the short abstract accordingly.

7. Please revise the Long Abstract (150-300 words) to include a statement about the purpose of the method. A more detailed overview of the method and a summary of its advantages, limitations, and applications is appropriate. Please focus on the general types of results acquired.

We have edited the long abstract as requested.

8. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

SI abbreviations has been used.

9. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Thank you for noticing this. We have corrected the spacing after numbers.

10. Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Statement has been moved

11. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Text was revised

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All the companies name identified have been removed, and the information included in the Table of Materials and Reagents. We did include the name of the instrument and proprietory software in Data Analysis, as this program is unique to how we calculated nitroxide concentration, however we also included alternative methods since the protocol does not require the specific spectrometer.

13. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

Numbering was adjusted

14. Line 121: Please specify the concentration of NaOH solution used.

Concentration of NaOH was added

15. Lines 148, 162-169, 214-217, 267-270, 276-279: Please write the text in the imperative tense in complete sentences.

Text was revised

16. 1.1.12: Does “KHB buffer + 100 µM DTPA” refer to the KHB containing 100 µM DTPA?

Please specify.

Yes - it appears now in the manuscript as KHB containing 100 µM DTPA

17. 1.3.1: Can this step be simply deleted? 1.3.2 referred to the specific steps.

Step has been deleted

18. 2.1.1: Please mention how animals are anesthetized and how proper anesthetization is confirmed.

A description of anesthesia has been added

19. 2.1.6, 3.1.7, etc.: Please specify the EPR conditions used in this step.
EPR conditions were added

20. 2.3.2: Please specify the volume of blood or BALF added.
Volume was specified

21. 3.1.1, 3.2.1, 3.3.2: Please refer to specific steps.
This part has been revised

22. 3.1.2: Please describe how to homogenize the tissue.
A description has been added (3.2.2)

23. 3.1.9: Please specify what is incubated with CMH.
In the referenced section 3.2 on fresh lung tissue, we have restated in 3.2.9 that the lung homogenate was incubated with CMH in the presence of several inhibitors

24. 3.2.2, 3.3.3: What is used to cut?

We used a single edged blade to cut tissue; this has been added to the protocol.

25. 3.2.7: Please specify the centrifugal speed.

*We added the mini table centrifuge speed (*8,500 *rpm*) (3.1.6)

26. Please include single-line spaces between all paragraphs, headings, steps, etc. After that, please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Text format has been revised as requested

27. Please discuss Figure 3C-D in the protocol.

Figure 3 has been modified to show the tracing of the CMH radical and the PTFE tubing utilized, and stated in the protocol step 1.3.1

28. Please discuss Figure 6C-D in the Representative Results.

Figure 6 has been modified and the results discussed in the representative results section
29. Line 479: Should it be Figure 5C, D?

Figure 5 has been modified

30. Please shorten the figure legends. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

Figure legends have been revised as instructed

31. Discussion: Please rephrase/revise to explicitly discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

Discussion has been edited to discuss critical steps, troubleshooting and limitations.

32. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis).

Format has been changed

33. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.
34. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

References have been revised

Reviewers' comments:

Reviewer #1:
Query 1: In presented manuscript authors describe the methods of detection superoxide using EPR and different hydroxylamine probes. Unfortunately, in manuscript authors show some flaws in logic:
In abstract authors state that EPR is "direct" and "specific" method for detection of ROS (superoxide in the case of this paper). However, presented in the paper method of superoxide detection is neither direct nor specific. First, authors do not detect superoxide directly but use hydroxylamine reaction with superoxide and detect the product of this reaction - nitroxide. This

approach does not have any difference compare to, for example, hydroethidine/fluorescent approach (detection of product of hydroethidine reaction with superoxide by fluorescence). Second, it is not specific method, authors use an addition of SOD to discriminate between superoxide specific and superoxide unspecific oxidation of hydroxylamines to corresponding nitroxides. I highly recommend rewriting manuscript according to logic.

We have edited the text to more accurately describe the advantage of utilizing EPR for the measurement of oxygen radicals. We acknowledge that EPR is not detecting superoxide itself due to the short half-life time of this radical, but instead, the reaction of superoxide with the EPR probe forms a nitroxide radical which is stable and thus detectable by EPR. We contend that EPR is a direct method to detect free radicals unambiguously, unlike hydroethidine (DHE). DHE requires a two-step reaction to detect $O_2^{\bullet -}$ through the formation of a free-radical intermediate followed by HPLC or relative fluorescence to detect the product; the reagent can be oxidized by other species and is subject to auto-oxidation.(J. Zielonka, B. Kalyanaraman / Free Radical Biology & Medicine 48 (2010) 983–1001). We have edited the manuscript to improve accuracy.

Query 2: As was said previously, authors use a SOD addition to elucidate superoxide-based oxidation of hydroxylamine to nitroxide for detection of superoxide production by biological milieus. In contrary, in mitochondria-based experiment authors directly show that addition of SOD "minimally affects" (statistically?) the increase of nitroxide signal (Figure 1C). Therefore, in this experiment authors show that hydroxylamine conversion to nitroxide is not happens due to oxidation by superoxide. Direct electron transfer from hydroxylamine to components of mitochondria should be considered for this experiment. This part of manuscript must be rewritten stating that no actual superoxide detection can be detected in this system but the mitochondrial redox state is detected, according to a logic of the rest of the paper.

We recognize that the exclusive use of a cell-impermeable SOD, which will not be able to react with cytosolic or mitochondrial superoxide, has raised confusion for this Reviewer, thus we have edited the manuscript to clarify the experiments and conclusions. We have also added supporting data with the cell permeable PEG-SOD to demonstrate the ability to differentiate superoxide generated in the extracellular vs. intracellular compartments using impermeable and permeable SOD. CMH was developed and is widely used as a superoxide-selective probe, though it does have the potential to react with other free radial species, necessitating the inclusion of DTPA as a metal chelator to prevent its reaction with metal ions. PEG-SOD will react with cytosolic but not mitochondrial superoxide. It is well established that mito-TEMPO-H, with the same targeting properties of MitoSOX, accumulates in the mitochondria and thus will react with mitochondrial superoxide (Dikalov et al. 2011). . To avoid confusion or inaccuracy, we have described the product as nitroxide when presenting the total CM$^{\cdot}$ signal and mitochondrial probe mito-TEMPO$^{\cdot}$ and reserve superoxide specifically for the SOD inhibitable component. As illustrated in the experiments in Figure 6B using lung tissue, one can also interrogate the source of superoxide with different enzymatic inhibitors.

Query 3: Authors do not provide any statistical description of data evaluation, statistical analyses has to be described and p values assign for all the data obtained.

Response 3: The project was designed to demonstrate methods rather than perform statistical analyses between treatment groups, thus though we have not included the statistical analyses and p values to the figures.

Query 4: Authors must check all mathematical calculations of concentration through out of the paper, especially in following cases:

We apologize for the errors and appreciate the careful review. We have carefully reviewed the calculations, including the specific examples identified by the Reviewer.

1) Line 151: MW is wrong.

We have corrected this mistake.

2) Lines 168-169: Stock solution concentration is not equal to 10 mM, compare to line 161.

The CPH and Mito-TEMPO-H concentration were corrected.

3) Wrong mathematical statement in line 227

Corrected

4) There are many inconsistence in actual prepare concentration trough out of paper.
There are no description of stock solution/buffers preparation procedure for lines 156, 343, 363-364

Buffers and stocks preparation were added

Query 5: There is no protocol for lung collection indicated in lines 341, 374, 408.

A description of lung collection was added

Query 6: Line 414 - no description of "tissue cell"
A description was added
Query 7: Something wrong with reference numbering, it is starting from 16 in line 275, please, correct.

Reference numbering started from the introduction

Query 8: SOD-based control date values must be included in Figures 4, 5, 6, 7

We have rearranged the presentation of the data and now include the SOD treatments only in Figure 6.

Query 9: Line 526: CAT1H compound is not described previously, please, give the name and description or reference

CAT1H name was added

Query 10: Line 539: Add description of SOD2

A description was added

Query 11: Line 508: Which component of CP was used?
We are not clear on the Reviewer's question. In the EPR spectra, we detected overlapping CP· signal and ascorbic acid radical signal. The data reported is the CP· component in the spectra. Using the SpinFit, one can distinguish the concentration or the Spins from spectra arising from multiple radicals

Query 12: In Table at he end of manuscript buffer DMEM is indicated, I was not able to find where his buffer was used.

DMEM is the cell culture media used for RAW 264.7 cells. DMEM was mentioned in line 176

Reviewer #2:
Article summary:
This is an article from Hanan Elajaili et al. describing a protocol using cyclic hydroxylamines and EPR to measure superoxide in vitro and ex vivo. The method is based on the oxidation of a cyclic hydroxylamine by the superoxide anion radical leading to an EPR active nitroxide radical that can be quantify by EPR. The methods is highly sensitive but suffer from a low selectivity as the hydroxylamine can be oxidize by many other species. Therefore, great care and appropriate controls are required.
Such protocol certainly deserve publication in JoVE but the manuscript contains mistakes that have to be corrected before being suitable for publication.

Major concerns:

The Reviewer noted multiple inaccuracies in the protocol; we regret these errors and appreciate the careful review that identified them. We have reviewed every calculation to ensure accuracy. Please see notes for the specific problems identified. Based on extensive publications, we propose that EPR is not highly sensitive, as compared to fluorescent probes, but is specific and allows for the quantitative measure of free radicals. The CMH probe is selective, though admittedly not exclusive, for superoxide, with no reactivity for hydrogen peroxide (S. I. Dikalov et al. 2004) It is commonly described as a superoxide selective probe. We have used nitroxide in

the y axis to reflect total CMH radical concentration, acknowledging that it may also include other superoxide-derived ROS and not exclusively the superoxide radical itself.

Query 1: L168. 5 mg of CPH in 1 mL will give a solution of 20 mM and not 10 mM, should all the finding using a wrong concentration of stock solution being corrected or it is a typo in the manuscript ?

Thank you for noting this mistake, which has been corrected.

Query 2: L190. 500 µL of KHB + 15 µL of SOD + 12.5 µL of CMH (10 mM) + 40 µL of PMA will not give a final concentration of CMH of 0.25 mM as stated nor 30 U/mL of SOD. Such inaccurate calculation repeats many time in the manuscript, please check and correct everywhere in the manuscript to ensure scientific rigor.
The step 1.1.1 in the protocol was corrected to read:

1.1.1. *Add KHB containing 100 µM DTPA to each well, and treat in a total volume of 500 µl with the following*

Query 2: How the nitroxide concentration is calculated by EPR? Please give details, is it using a calibration curve based on the double integration or based on the intensity of one peak.
EPR spectra were simulated using SpinFit, a program incorporated in the software of the specific spectrometer used, and the concentration was obtained using SpinCount. Alternatively a calibration curve of a stable nitroxide such as 4-Hydroxy-TEMPO (TEMPOL) or 3-Carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl can be made and the concentration can be obtained by comparing the intensity of the signal from the sample and the standard. These options have been clarified.

Query 3: L227 at l160 the stock solution is 10 mM, at l227 it is 9.5mM.
The stock solution has been presented as 9.5 mM rather than rounded up to 10 mM.

Query 4: L435-438 The sentence is of not very clear, please reformulate. The level of the blank is never indicated, it would be interesting to show the time course of the appearance of the EPR signal with the probe alone under the same condition or at least the level at the same time of measurement.

The sentences were revised to be clearer. We did not perform the time sweep experiment to monitor the increase in the signal over time. Instead, we present the end point measurements after 50 min incubation time period. The incubation time was based on pilot experiments not included in the protocol to ensure the reaction was complete. The EPR signal with the probe alone under the same condition it has been always used as a control and is now included.

Query 5: Having graphs showing the concentration of superoxide is misleading. For example, Fig4D shows a concentration of superoxide in the blood of about 25 µM. This is not really the concentration of superoxide in the blood (steady state) but rather the concentration of

nitroxide radical accumulated for a certain time from the reaction of the hydroxylamine with superoxide.
It has been clarified in the figure legend and in the representative results that the concentration reported is the concentration of the nitroxide that accumulated in blood or BALF under the experimental conditions.

Query 6: The reason for measuring the EPR at 77k is not given. Please explain.

We evaluate samples both at room temperature and at 77K. A central goal of this JoVE manuscript is to demonstrate a practical method to collect samples and store them at -80 C and collect data at 77K, allowing for a more convenient efficient work flow and ability to transfer samples from other labs to an EPR facility. Another advantage is the ability to test a larger volume of sample. At room temperature, the measurements are performed in EPR capillary tubes while at 77k, the larger volumes frozen in the EPR tubing allow for improved signal to noise, and thus sensitivity.

Minor Concerns:
Query 7: L160. Degassed should be replace by deoxygenated.
Degassed has been replaced by deoxygenated.

Query 8: L119. The name of DTPA is wrong.

The spelling of DTPA was corrected

Query 9: mito-TEMPO-H is some time written as mito-Tempo-H. TEMPO is more commonly used than Tempo.

We now consistently used mito-TEMPO-H

Query 10: L267. Correct the time constant.

The time constant is corrected

Query 11: L284 100 µL of heparin (concentration?).
We *used 100 µl of 1000 USP units per ml (Sagent Pharmaceuticals, Inc.). Concentration was added and the product was added to the table of materials.*
Alternatively we used a syringe coated with heparin containing DTPA 100 µM

Query 12: L324 "load blood" this part is superoxide in BALF.

Corrected

Query 13: L405 100 µL of CPH (presumably from the 10 mM solution?) injected at a dose of 20 mg/kg would give a mouse of about 11 g, is it correct?

To prepare CPH stock solution 5 mg was dissolved in 1ml filtered and deoxygenated 50 mM phosphate buffer which gives 22 mM . 10 mM was a typo. 100 µl of 5 mg/ml of CPH stock was giving to mice with an average body weight of 25 g for 20mg/kg dose. This was stated now in the manuscript

Query 14: The Figure 3D is not clear, it would be very valuable to replace it.

3C and 3D have been removed and replaced with a better photo.

Query 15: In general, the fraction of nitroxide generated from the superoxide is only a fraction of the total signal. One can wonder whether this method is adequate for measuring superoxide? It is also true that measuring superoxide radical is extremely challenging and other techniques have their own drawbacks.

We agree that the extracellular superoxide was a small portion of the total nitroxide signal, shown by the cell-impermeable SOD1 inhibitable component. We did not directly determine the contribution of intracellular superoxide and other investigators have also demonstrated that CMH is relatively selective for superoxide. That being said, we have adjusted the protocol to reflect the more conservative accurate interpretation. We also have presented a protocol to identify the other species contributing to the total nitroxide signal which most likely generated downstream results of superoxide being generated in the system.

Reviewer #3:

Manuscript Summary:

The manuscript entitled "Superoxide Detection in in vitro and in vivo Models Using Spin Probes and Electron Paramagnetic Resonance (EPR) at Ambient Temperature and 77 K" by H. Elajailli et al. present different methods for the detection of ROS using hydroxylamine spin probes and EPR in cells and tissues. The authors have published a large number of articles using these methods and have thus a high expertise regarding their application. However, the manuscript does not satisfy educational standards expected in JoVE because some interpretations are not supported by the presented results.

We appreciate this comment and have extensively editing the manuscript to refine the interpretations of the data and ensure it meets the educational standards of JoVE.

Major Concerns:

Query 1 and 2: The limitations of the technique should be much more clearly stated and appropriate controls added (especially with SOD-PEG). I agree with the statement in the abstract that EPR detection using spin probes has many advantages but that appropriate controls are critical to ensure accurate interpretation of the results. The discussion lists rather clearly the limitations of the technique and precautions for accurate interpretations. On the

contrary, the title, abstract, introduction, method, and result sections are much more ambiguous by insisting on superoxide detection and determination of superoxide concentrations. Therefore, unexperienced readers may overestimate the specificity of the detection (as often seen in the literature). Cyclic hydroxylamine spin probes react not only with superoxide but also with many other radicals and metal ions. Depending on the cell or tissue type and condition, the level of nitroxide radical signal due to superoxide may vary. Interferences due to hydroxylamine oxidation by enzymatic processes have been reported (for example, with photosynthetic electron transport chain in Kozuleva et al. Free Radic. Biol. Med. 89 (2015) 1014-1023; with cytochrome c oxidase in Chen et al. Biochim. Biophys. Acta 970 (1988) 90126-90127). Controls with cell-impermeable SOD only reports on extracellular superoxide production and are not sufficient to decide on the origin of the nitroxide signal remaining after SOD treatment. Typically, cell-permeable SOD-PEG controls are required to attribute part of the signal that was not inhibited by SOD to intracellular superoxide using the procedure applied by the authors in a previous study (reference 19). It would be helpful to the reader to give a list of potential interferences with appropriate controls to rule them out as a table in the manuscript.

Query 2: In accordance with the previous remark, the title of the article should be changed to "ROS detection in in vitro and in vivo models...". Also the titles of protocols at lines 172, 219, 247, 280, 282, 307, 327, 338, 340, 373, and 403 and the titles of result sections at lines 419, 449, 462, 465, and 484 should be more accurate and refer to "ROS detection" instead of "superoxide detection", unless specific controls including SOD-PEG are added. The main text should be corrected in a similar way throughout.

We thank the Reviewer for this important point. While we want to emphasize the established function of CMH as a superoxide selective probe that does not react with hydrogen peroxide, we also do not want to overstate the signal as indicating superoxide without confirming the SOD-inhibitable component of the signal. We have therefore changed the nomenclature to use nitroxide radical rather than superoxide in the y-axis of the figures unless we are representing only the SOD inhibitable signal, and modified this in the text. We recognize that other radicals can react with CMH, and have included the table in the discussion with different controls, as suggested above. We also included the important point raised that enzymatic processes such as cytochrome c oxidase can result in oxidation of hydroxylamine and recommended the researchers to be cautious in Interpreting the results.

Query 3: Short Abstract (Lines 38-40): Even though the detection of free radicals by EPR is direct, when spin probes are used to detect ROS, it is an indirect method of detection of ROS. The proximity of the two first sentences in the short abstract is thus misleading. I advise they are changed to "The use of different types of spin probes coupled to electron paramagnetic resonance spectroscopy (EPR) allows for the detection of reactive oxygen species (ROS), including superoxide, in specific cellular compartments, provided appropriate controls are performed to ensure accurate interpretation of the data. Storage of samples..."

Thank you for the suggestion. The abstract has been revised.

Query 4: Abstract (lines 46-52): As in the short abstract, this section is misleading. EPR is unable

to measure directly ROS radicals at physiological levels due to instability and lack of sensitivity. The use of spin probes (or spin traps) is an indirect method of detection of ROS and it should be clearly stated.

Response 4: We acknowledge that EPR is not detecting superoxide itself due to the short half-life time of this radical, but instead, the reaction of superoxide with the EPR probe forms a nitroxide radical which is stable and thus detectable by EPR. We contend that EPR is a direct method to detect free radicals unambiguously

Query 5: Abstract (line 56-58): Add SOD-PEG pretreatment to allow for intracellular superoxide detection. It is excessive to talk about "superoxide concentration" when intracellular or intramitochondrial detections are performed without the proper controls that allow exclusion of interfering processes.

We have added additional data to demonstrate the use of SOD-PEG to designate cytosolic superoxide. It is well established that mito-TEMPO-H, with the same targeting properties of MitoSOX, accumulates in the mitochondria and thus will react with mitochondrial superoxide. Previous study shows that SOD2 overexpression significantly attenuated measurements with mitoTEMPO-H (Dikalov et al. 2011) and this control has been described in the discussion.

Query 6: Introduction (line 91): Kinetic comparison with spin traps is misleading. Reaction rates of hydroxylamine spin probes with superoxide are in the range of 10^3-10^4 M-1.s-1. Antioxidant enzymes as SOD (k ≈ 10^9 M-1 s-1) will outcompete the spin probe easily, so any variation in the level of antioxidant enzymes will ruin accurate determination of superoxide concentration with spin probes.

We agree with the Reviewer that the reaction between superoxide and SOD is faster than superoxide with the spin probes, however, high concentrations of the spin probes detect superoxide in biologic settings. These methods can be used to determine the ROS production in specific pathophysiologic conditions associated with altered production or antioxidant defenses as shown in this study, or in experimental conditions designed exclusively to test the production of superoxide from a designated source.

Query 7: Introduction (line 103): Additional references of applications of spin probes in vitro should be given here.

References have been added.

Query 8:Results (line 428): The attribution of remaining signal to intracellular superoxide is abusive. It certainly is due to intracellular nitroxide but nothing in the experimental procedure support links it to superoxide. If the authors have additional elements to support this attribution (such as additional experiments with SOD-PEG), they should add them in the manuscript. Otherwise, the interpretation must be revised and Figure 1B removed. (Same remark for line 444).

As discussed above, we have substituted nitroxide for superoxide when indicating total CMH signal, and reserved superoxide only for the SOD or SOD-PEG inhibitable signal.

Query 9: Results (line 435): The attribution of the signal of mito-TEMPO-H after SOD treatment

to mitochondrial superoxide and derivation of superoxide concentration are abusive. It has been shown that the nitroxide derived from mito-TEMPO-H is reduced in the mitochondria (Dikalova et al. Redox Biol. 4 (2015) 355-362), which is likely to interfere with the measurement of absolute superoxide concentrations, especially rate of bioreduction vary between conditions. Moreover, the experimental conditions do not allow exclusion of interference from other ROS. Thus, Figure 1D should be removed.

Response 9: We have attributed the mito-TEMPO-H signal as mitochondrial superoxide. The probe is designed to accumulate in the mitochondria. Antimycin A (AA) a known inhibitor of mitochondrial electron transport chain (mETC) complex III has been used to induce superoxide production in the mitochondria and the mito-TEMPO$^{\cdot}$ signal was increased upon the stimulation with Antimycin A. We based our interpretation based on a previous study that shows that SOD2 overexpression significantly attenuated measurements with mitoTEMPO-H (Dikalov et al. 2011). We agree with the reviewer that the signal after SOD treatment is misleading and have removed this component of the experiment. As discussed in the referenced manuscript (Dikalova et al. Redox Biol. 4 (2015) 355-362) *the nitroxide signal from mito-TEMPO-H may underestimate the exact superoxide generation in the mitochondria due to the bioreduction of nitroxide radical.*

Query 10: Results (Lines 454-461): It should be made clear that only extracellular superoxide is measured in blood and BALF.

Since we treated the whole blood and BALF containing cells, CMH will detect both intracellular and extracellular ROS. We removed the SOD treatments in these figures and thus did not present data for extracellular superoxide.

Query 11: Results (lines 466-473): Do the authors assume the homogenate contains intact cells? If so, they should specify that only extracellular superoxide is measured in fresh lung tissue homogenates using their protocol. Is there any difference in the results when SOD-PEG is used instead of SOD?

We understand the importance of this question. Upon reflection, we cannot be confident whether the SOD inhibitable signal represents only extracellular superoxide in homogenized and frozen samples since it is also possible that cell membranes are damaged. We note that if this information is desired, this can be more accurately assessed using the in vivo spin probe delivery shown in Figure 7 with permeable or impermeable SOD. To elucidate the origin of the nitroxide signal generated, we also presented a protocol to use scavengers and enzyme inhibitors to identify the enzymatic source of the ROS or define the contribution of different radicals. (Figure 6 and Table)

Query 12: Results (from line 499): It should be explained here or in the discussion why CPH is preferred over CMH for in vivo experiments.

It is unknown if CMH can be safely administered to animals while injection of CPH probe has been reported to be non-toxic, thus we selected CPH for in vivo experiments. We have clarified this in the representative results.

Query 13: Discussion (lines 591-596): The reader should be warned that adaptation of the protocols described here is possible but that a thorough investigation of the identity of ROS involved in the formation of the nitroxide needs to be performed using appropriate antioxidants before attribution of the signal is performed in a new system. Besides, additional controls need to be performed to prove that the level of endogenous antioxidant enzymes (SOD) and the reduction potential of the tissue are similar between conditions when concentrations of superoxide are derived and compared.

Thank you for these comments. We agree with these points. We expect that alterations in endogenous antioxidant enzymes, and redox state will change the levels of specific ROS under disease conditions and propose that EPR is a useful method to evaluate this problem.

Query 14:Legend of Figure 1 + Figure 1 (lines 606-611): Reference to total superoxide, intracellular superoxide, and mitochondrial superoxide are abusive as discussed above. Labels of y-axis in Fig. 1B and 1D also are, so they should be removed.

As discussed several times above, we will improve accuracy and rigor, using nitroxide in the y axis to reflect total CMH radical concentration, and use superoxide to reflect only the SOD inhibitable component.

Query 15:. Figure 2: The y-axis should be labeled "Nitroxide" and not "Superoxide".

As discussed above, we agree and have altered the y axis labels.

Query 16: Figures 4, 5, and 6: The values of the SOD treated samples for each condition should be displayed in the figures.
figures have been modified to better represent the protocol

Minor Concerns:
Query 17: Line 168: check mass of CPH to be added.
To prepare CPH stock solution 5 mg was dissolved in 1ml filtered and deoxygenated 50 mM phosphate buffer which gives 22 mM . 10 mM was a typo. 100 µl of 5 mg/ml of CPH stock was giving to mice with an average body weight of 25 g for 20mg/kg dose. This was stated now in the manuscript

Query 18: Protocols (line 186): Control with SOD-PEG should be added
An additional data regarding the effect of SOD-PEG on the signal was added

Query 19Protocols (line 199): It is not clear what is done with this buffer sample.

Response 19: We have clarified that EPR measurements were performed on the buffer.

Query 20: Protocols (line 201). It is not clear what is done with this cell sample.

Response 20: We also clarified that EPR measurements were performed on the cells.

Query 21 : 21. Protocols (line 206): It is not clear whether the sample is that prepared at line 199, at line 201, or both.

Both and that was clarified in the protocol

22. Protocols (line 224): SOD treatment is missing here.
SOD treatment was removed from the figure.

Query 23:Line 270: "1.28 ms" instead of "1.28 m".
The typo was corrected

Query 24: Line 308: PBS contains DTPA, not the syringe.
Corrected

Query 25: Line 343: Tris-EDTA buffer containing sucrose does not appear in the reagent list nor in the table of materials.
Buffers preparation was added

Query 26: Protocols (line 362): Reagents used for the controls and their preparation must be added to the reagent list.
: Stocks preparations were added

Query 27: Line 379: Specify that the weight of each piece of lung tissue must be recorded independently.
Specified

Query 28Line 381: It is not clear that the first piece of lung tissue cut at step 3.2.2 is used here and not both.

Response 28: clarified

Query 29: Protocols (Lines 381 and 384): What is meant by "mix"? Is homogenization of the tissue performed?

No homogenization was performed.

Query 30: Line 386: The tissue weight of the second piece of tissue was already recorded at line 379. *Corrected*

Query 31: Lines 397-401 should be placed in paragraph 3.2.2 at line 377.

Response 31: step was modified

query 32 : = Line 405: How is CPH solution for in vivo injection prepared?

Response 32 :A description and more details about the preparation has been added

Query 33 : Lines 437-438: Verb is missing in the sentence.
Thank you the sentence was edited

Query 34 : Line 479: Give figure number.
Figure was modified

Query 35: Lines 481-483: The sentence about frozen tissue should be moved to the next section dedicated to these type of samples (near line 497).
The sentence moved
Query 36: Line 489: A full stop is missing after concentration.

Response 36: *Thank you. The sentence was fixed*.

Query 37: Lines 491-492: Verb is missing in the sentence.

Response 37: The sentence was edited

Query 38: Legend of Figure 3 (line 626) + Figure 3: The title of the legend (referring to 77K) does not match part 3B of the figure (room temperature spectra).

Response 38: figure 3 B was removed to prevent confusion and since a typical CM· spectra at room temperature was presented in figure 1

Query 39: Lines 635-636: Verb is missing in the sentence.
The sentence was edited

Query 40 Legend of Figure 5B (line 646) and Figure 6B (line 652): Explain how superoxide component is derived from the data in the legend.

Superoxide component was derived from subtracting the signal in presence of SOD from the signal in the absence of SOD. The figures have been modified.
Query 41: Figure 5C: CMH is present in all conditions. Please clarify x-axis label to reflect this fact. The value for the first condition in figure 5C does not match the values in Figure 5A. Why? Please clarify the legend.

different tissues from different mice were used and we observed variability due to the heterogeneity in lung injury with bleomycin. Also the test using different inhibitors was done at 37 C and we are comparing to effect of the inhibitors to the values in figure C not A. new data has been presented in the revised version.

Query 42 :Reference 12 on line 726 is incomplete.
Corrected

Query 43: Authors should consider including heparin (from section 2.1.1), catalase, and the tissue cell in the table of materials. Maybe they should give some details about the characteristics of the EPR spectrometer needed.
Information was added

Reviewer #4:
Manuscript Summary:
This manuscript presents practical methods for detection of superoxide using cyclic hydroxylamine spin probes and EPR in different cellular compartments in vitro and different fluids and lung tissue in vivo, at both room temperature and 77 K. The topic of the article is of great interest, the protocols are well described and allow other researchers to replicate the experiments, however, there are a few minor issues to clarify.

Minor Concerns:
Query 1: In methods, additional information that should be included is about how the EPR signal calibration was done. Was a calibration curve performed with CM* or CP*? How should the preparation of these stable radicals be done? What concentrations should be used?
EPR spectra were simulated using SpinFit, a program incorporated in the software of the spectrometer used here, and the concentration was obtained using SpinCount. Alternatively a calibration curve of a stable nitroxide such as 4-Hydroxy-TEMPO (TEMPOL) or 3-Carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl can be made and the concentration can be obtained by comparing the intensity of the signal from the sample and the standard.
CM and CP cannot be used for obtaining a calibration curve.

Query 2:please confirm in the preparation of the spin probes the pH of the phosphate buffer. This solution is not included in the list of materials. PBS is also not included.
The pH of the phosphate buffer and buffer preparation as well as the reagents were added to the methods and list of materials.

Query 3:Please clarify the number of animals used in Figure 4. Panels A and B show data of 4-5, but C-F panels only show data of 2 animals per group. Samples were not obtained from the same animals? The legend does not show the n.
In Figure 4, each point in the graph represents a different mouse. The same mice were used for both Fig 4A and Fig 4B, though in some mice, collection of the blood was unsuccessful, accounting for the variable n. The figure has been modified and now only includes the total nitroxide signal.

Query 4:Discuss the choice of CPH for in vivo injection. CMH was used in all other in vitro and ex vivo experiments. Is there any advantage to using the CPH in this case?

It is unknown if CMH can be safely administered to animals while injection of CPH probe has been reported to be non-toxic; therefore we selected CPH for in vivo experiments. We have clarified this in the methods.

Thank you for the editorial review of our submission. We have addressed each of the queries below and edited the manuscript accordingly.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you – we have carefully proofread the manuscript to correct spelling and grammar

2. The short abstract is over the 50 word limit.

Short abstract has been revised as requested

3. The protocol has been rearranged. Please ensure that all references are numbered based on their appearance in the manuscript.

References have been checked

4. All protocol steps are renumbered. Please ensure that all protocol steps are numbered correctly when they are referenced. For example, steps 1.1.11 and

1.1.12 are referenced in line 279, however, they are numbered different, so please correct the numbering.

Thank you. This is an important point. We have checked the numbering

5. Please do not highlight notes for filming.

We were unclear about this instruction as we understood you did want us to highlight the areas relevant to filming. Therefore we have submitted two versions of the manuscript: one with the part of the protocol to be filmed highlighted and one without highlighting

6. Please remove all header in Representative Results.

All header were removed in Representative Results.

7. Please combine some short steps so that each step contains 2-3 actions.

We combined short steps when possible without interfering with subsequent referencing of steps

8. Please use standard SI unit symbols and prefixes such as µL, mL, L, g, m, etc.

We used the standard SI units, symbols and prefixes in the manuscript.

9. Step 3.1.1: Please mention how animals are anesthetized and how proper anesthetization is confirmed.

We have added that we confirmed appropriate anesthesia via toe pinch. We also mentioned that all animal studies were approved by the University of Colorado Denver Institutional Animal Care and Use Committee. We request that this statement be included with the animal work (in vivo) section in the protocol rather than before the reagents preparation.

10. Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

We have now specified this point in 4.3.2

11. For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

Mice do not undergo survival surgery.

12. Discuss maintenance of sterile conditions during survival surgery.

As stated above we did not do survival surgery

13. Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

We have clarified that mice are left attended until they regain consciousness after procedures.

14. Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

All mice were euthanized and did not undergo survival surgery.

15. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Speed changed to (xg)

16. Step 5.1: Please write this step in imperative tense.

Step was revised as requested