

Identification of a Free Radical Scavenger as an Additive for Lung Transplant Preservation Solution to Inhibit Coagulative Necrosis and Extend Organ Preservation

Durga Ganesh, Amanda Alonzo

Lynbrook High School, San Jose, California

Summary

Among the routine organs being transplanted, the lungs and heart deteriorate the fastest during transport from the donor to the recipient. Only 15–20% of cadaveric donor lungs are usable for transplant. Lungs can be preserved for transport in cold ischemic, hypothermic preservation solutions for only 4 to 6 hours. Extending lung preservation time would allow for greater organ transport distances, as well as for better assessment and repair of harvested organs. This research aims to extend the transport life of lungs in hypothermic preservation solution. Significant coagulative necrosis, a pattern of cell damage due to free radicals, develops in the donated lung during cold storage transport. We hypothesized that application of antioxidants can prevent free radical-induced cell damage; the goal of this study is to identify antioxidants that are effective in reducing free radical-induced damage to lungs over time. To accomplish this goal, several antioxidants were evaluated for preserving bovine lung cell morphology at two time points. Vitamin E and butylated hydroxytoluene (BHT) were found to be the most effective at arresting cell damage. We recommend further evaluation of different concentrations of vitamin E and BHT as additives to organ preservation solutions used today.

Received: November 13, 2014; **Accepted:** January 12, 2015; **Published:** February 12, 2015

Copyright: (C) 2015 Ganesh and Alonzo. All JEI articles are distributed under the attribution non-commercial, no derivative license (<http://creativecommons.org/licenses/by-nc-nd/3.0/>). This means that anyone is free to share, copy and distribute an unaltered article for non-commercial purposes provided the original author and source is credited.

Introduction

Organ transplantation is a growing component of today's health care. Although an average of 79 patients receive organ transplants each day in the United States, 131 patients are added to the waiting list (1). An estimated 21 people die every day awaiting a transplant due to this imbalance between the available donors and potential recipients (2). Between 1997 and 2007, there was an 11% increase in the number of candidates on the lung transplant list (3). Lung transplantation is only recommended for patients likely to die within 1–2 years of end stage lung diseases such as severe chronic obstructive pulmonary disorder (COPD), cystic fibrosis, pulmonary fibrosis, and pulmonary hypertension (4). However, only half of the patients on the lung transplant

waitlist receive the transplant (5).

Most lung transplants are from cadavers (6). In addition to factors such as donor health and hospitalization (7), the gradual deterioration in the quality of lungs during transport from the donor to the recipient contributes to the limited number of viable organ transplants (8). The standard method of preserving donor lungs in transit is through cold ischemia, in which blood flow is cut off and the organ is cooled in two stages (in situ and ex vivo) with a hypothermic dextran-electrolyte preservation solution such as Perfadex® (9,10,11). Techniques to extend the preservation time of donated lung tissue by continuous perfusion using a pulsatile perfusion machine is still under research (10). Thus, only 15–20% of cadaveric donor lungs are usable in the United States (12).

At present, routine cadaveric lung transport in hypothermia is limited to 4 to 6 hours (13), after which excessive cell damage by free radicals limits the chances of a viable transplant (14). During lung preservation, levels of free radicals increase, and the cells' capacity to scavenge them is compromised, thus leading to rapid deterioration of the organ (15). Highly reactive atoms erode the cell membrane, causing excessive cell damage. Antioxidants neutralize free radicals and stop the chain reaction of oxidative stress. Prior research has found that antioxidants appear to play a beneficial role during liver transplantation (16).

Cell damage was measured by evaluating coagulative necrosis in lung tissue. Necrosis describes the premature death of cells through autolysis due to external stresses (17). Coagulative necrosis is the distinct, morphological pattern of necrosis most commonly caused by severe ischemia (17). Coagulation is a result of protein denaturation, causing albumin to become firm and opaque. Under a light microscope, coagulated tissue stained with hemotoxylin and eosin will appear lighter in color, with fragmented or missing nuclei (17). Hemotoxylin is a dark purple stain that marks protein structures and the nucleus. Eosin is a pink stain that recognizes coagulated cells' cytoplasm; this lighter hue is due to the removed structures in the cytoplasm.

Butylated hydroxytoluene (BHT), vitamin A, vitamin E, lutein, vitamin C, melatonin, and biotin, which have been documented as effective antioxidants (18), were evaluated in this study and compared against ice. BHT is currently used in food preservatives to prevent the spoilage of fat and is the only synthetic preservative tested in this study (19). Vitamin A enhances the immune system, and vitamin C has been shown to be a powerful antioxidant (20, 21). The remaining preservatives (vitamin E, lutein, melatonin, biotin) were tested based

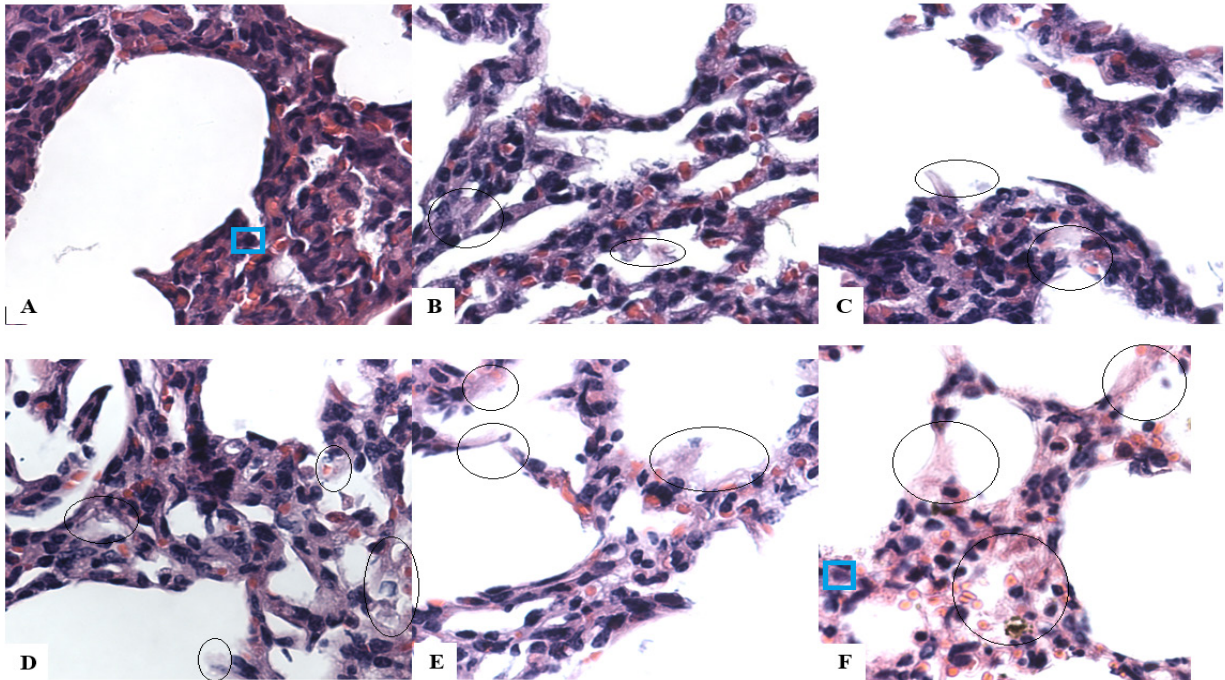


Figure 1: Representative images of lung tissue samples after 5 hours of preservation. Red blood cells are interspersed throughout the tissue. Areas marked by black circles indicate coagulative necrosis and cell deterioration. Images were taken at 400x. (A) Sample examined at the start of experimentation with no preservatives. Area marked by a blue square at the bottom right corner of sample indicates a representation of an intact nucleus. (B) Sample preserved in vitamin E solution. (C) Sample preserved in BHT solution. (D) Sample preserved in vitamin A solution. (E) Sample preserved in ice. (F) Sample preserved in vitamin C solution. Area marked by a blue square at the bottom left corner of sample indicates a representation of a washed out nucleus.

on their noted antioxidant properties from the literature (17). Saffola oil®, pure oil from natural safflower seeds, was the solvent for all fat-soluble antioxidants. Our study aimed to determine the effectiveness of these antioxidant additives in preserving lung tissue. We hypothesized that free radical-induced lung cell damage can be controlled by identifying an effective antioxidant additive to the preservation solution.

Results

Prior to any preservation (Figure 1a), the nuclei of the tissue sample were densely packed with a dark blue hue. This represented the initial health of the samples. A dark purple cytoplasm indicated intact eosinophilic structures without excessive protein dispersion.

The sample preserved in vitamin E solution for 5 hours (Figure 1b) had mostly condensed nuclei with a dark blue hue. Lighter colored cytoplasm indicated intact eosinophilic structures, although there was a slight clumping of protein structures.

The sample preserved in BHT solution for 5 hours (Figure 1c) had a lighter colored and shredded cytoplasm, indicating minimal leakage. There was uneven clumping of protein structures in the cytoplasm, varying the lavender hue throughout the sample.

The sample preserved in vitamin A solution for 5 hours (Figure 1d) had areas with missing nuclei, characteristic of coagulative necrosis. Lighter colored, shredded,

and faded cytoplasm indicated leakage and noticeably uneven clumping of protein structures in the cytoplasm.

The sample preserved in ice for 5 hours (Figure 1e) appeared similar to the vitamin A sample under the light microscope. However, the violet hue of the cytoplasm was a lighter shade of purple, indicating minimal leakage of eosinophilic structures into the cytoplasm.

The cytoplasm of the sample preserved in vitamin C for 5 hours was extremely light pink with heavy clumping (Figure 1f). This indicated heavy leakage of protein structures unevenly throughout the cytoplasm, a likely result of osmotic shock. Osmotic shock could be overcome in future studies by incorporating an isotonic (0.9%) saline solution into the vitamin C preservative solution.

Based on the morphological evaluation of lung tissue in preservatives after 0 hours, 3 hours, and 5 hours, vitamin E and BHT were the most effective additives to the preservation solution (Figure 2). Only preservatives with a rating over 10/20 after 3 hours of preservation were evaluated after 5 hours through a second round of testing (Figure 2). Figure 3 displays representative hematoxylin and eosin (H&E) stained slides of tissue after 3 hours of preservation.

When comparing the health of nuclei after 5 hours of preservation, samples in vitamin E and BHT had over 20% more intact nuclei than the sample preserved in ice (Figure 4). Vitamin E and BHT also had the least

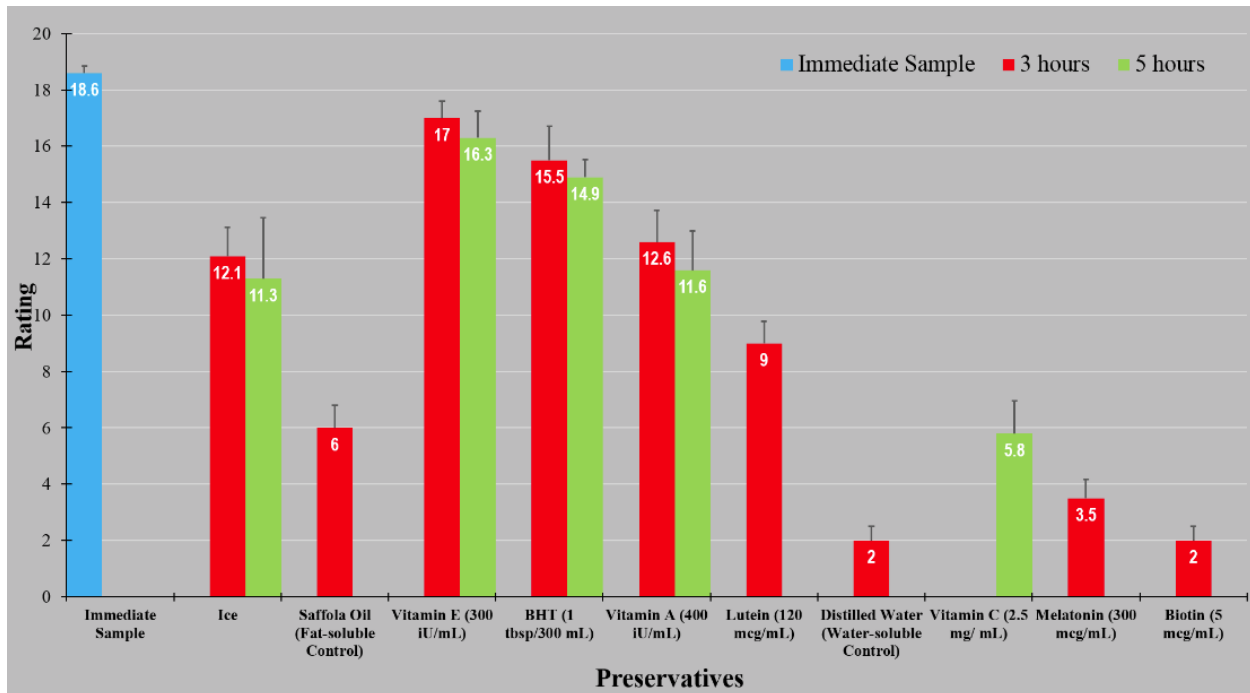


Figure 2: Cell health rating comparison. The average of the final ratings of 5 random samples per preservative. Error bars indicate standard deviation. Initial ratings were made after 3 hours of preservation. Only the preservatives with a rating greater than 10/20 for the 3 hour samples were evaluated after 5 hours through a second round of testing. Preservatives whose 3 hour ratings were below the threshold of 10/20 were not considered for evaluation at 5 hours. The only exception was vitamin C, which only had the 5 hour sample. Three hour evaluations for vitamin C were discarded due to systematic procedural error. This procedural error was detected early on in just the individual experiment with vitamin C, and it was avoided in all other samples. The samples with antioxidants fared better than the samples with plain solvents like Saffola oil® and distilled water.

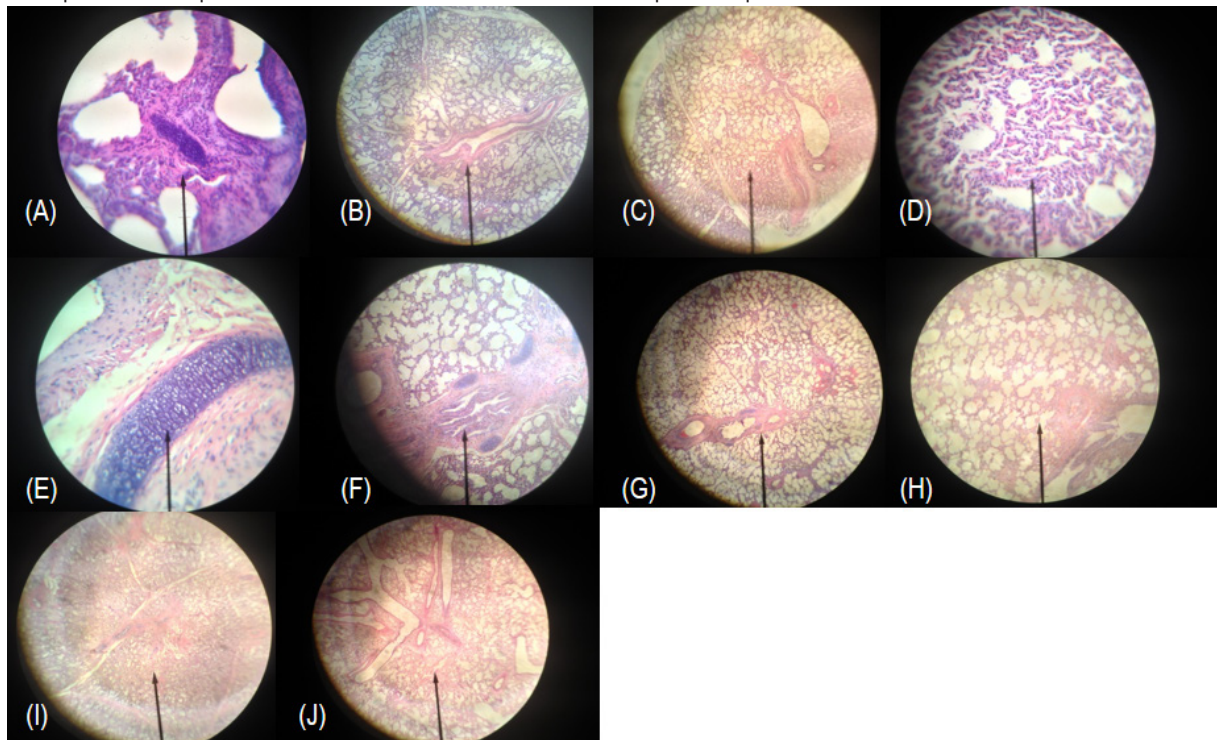


Figure 3: Representative images of lung tissue samples after 3 hours of preservation. Images were taken at 40x. (A) Sample examined at the start of experimentation with no preservatives. (B) Sample preserved in ice. (C) Sample preserved in Saffola oil®. (D) Sample preserved in vitamin E solution. (E) Sample preserved in BHT. (F) Sample preserved in vitamin A solution. (G) Sample preserved in lutein solution. (H) Sample preserved in distilled water. (I) Sample preserved in melatonin solution. (J) Sample preserved in biotin solution.

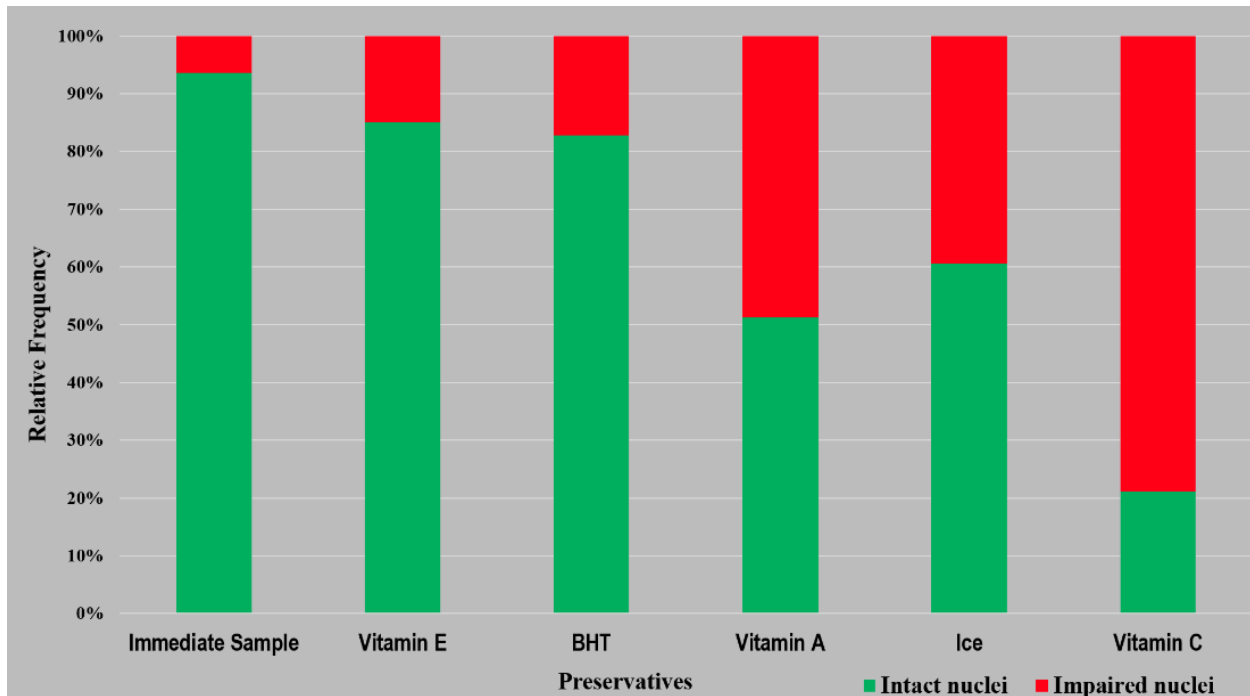


Figure 4: Nucleus health comparison. For each of the samples evaluated at 5 hours, the health of H&E stained tissue was quantitatively evaluated. Each nucleus in the sample was categorized as intact or impaired, and the relative frequency of each type of nucleus was identified for each preservative. Vitamin E and BHT had over 20% more intact nuclei than the sample preserved in ice.

sample variation and standard deviation (Figure 5). Vitamin E and BHT, with a standard deviation of at most 0.94, consistently and significantly reduced cell damage compared to the other preservatives. After 5 hours of immersion, vitamin E was the most effective preservative, closely followed by BHT and ice (Figure 2).

Our results show that the solvents (Saffola oil® and distilled water) do not contribute to the effectiveness of the antioxidant (solute). At 3 hours, water-soluble preservatives were at least as effective as distilled water, the control for the water-soluble preservatives, in maintaining cell structure (Figure 2). Similarly, fat-soluble preservatives were more effective than pure Saffola oil® (Figure 2).

Discussion

Overall, oil-soluble preservatives were more effective than water-soluble antioxidants. Deducing the reason behind the effectiveness of the oil-soluble preservatives can be investigated in a future study by evaluating samples from different depths of tissue. If oil-soluble preservatives penetrate deeper into tissue than water-soluble preservatives, then this may be a potential reason for the increased effectiveness of oil-soluble preservatives.

These experiments were carried out on tissue samples from a single bovine lung specimen. Future studies with samples from several bovine lung specimens would produce results with higher confidence.

In this study, distilled water was used as the solvent for water-soluble antioxidants, which subjected the samples

to osmotic shock. The low osmolarity of water relative to lung tissue formed a hypotonic solution, causing osmotic shock. Future studies can test distilled water and an isotonic (0.9%) saline solution, with and without antioxidants, to account for each variable specifically.

Arriving at the optimal concentrations of preservatives was a challenge due to the lack of significant prior investigation into natural preservatives. For consistency, the manufacturer's highest recommended dosage for each antioxidant supplement was used (Figure 2). To optimize preservative concentrations, this study recommends repeating preservative evaluations with different concentrations of vitamin E and BHT, the two most promising additives.

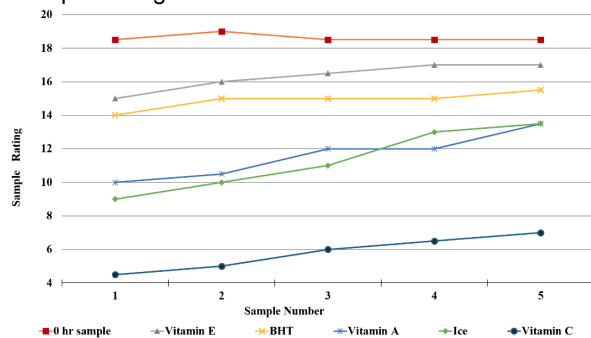


Figure 5: Cell health rating per individual sample after 5 hours of preservation in antioxidants. The individual ratings of all samples measured after 5 hours of preservation. As the sample rating decreased, variability within the sample increased.

A systematic error was detected with the evaluation of the tissue samples preserved in vitamin C for 3 hours. This specific slide set was processed in a different batch than the other samples during the H&E staining process. The visual observation of the colors of cell components was a key factor in comparing cell health. To avoid any bias or error due to possibly uncontrolled variables, the histopathology of vitamin C at 3 hours of preservation was not evaluated. However, loss of this data did not impact the overall results, as the 5 hour preservation rating of vitamin C was the poorest of all additives for this time point (Figure 2).

Our research is supported by existing work, which shows that vitamin E has exhibited protective preservative properties on rat kidney tissue (22). Rat kidneys flushed with Marshall's Citrate and added vitamin E, instead of Marshall's Citrate alone, exhibited better protected metabolism and tubular ultrastructure (22). In addition, BHT is a popular preservative used for fatty foods (19). Thus, its preservative properties on lung tissue are supported by its current preservative use in the food industry. Another study indicated potential benefits of using antioxidant therapy for both the donor and recipient of liver transplants (23). Our research conclusions corroborate with findings that suggest the addition of free radical scavengers to organ preservation solutions as a means of protecting endothelial cells against cold ischemia and reperfusion injury following transplantation (24).

For future studies, water-soluble preservatives should be retested in 0.9% saline solution to reaffirm poor preservation by the water-soluble antioxidants. Also, the optimal concentrations of vitamin E and BHT for minimal cell damage should be determined. The chosen additives can be added to and tested with commercial perfusion solutions such as Perfadex®, which contains dextran 40, a colloid component, and other aqueous elements in lower concentrations, including Na⁺, K⁺, Mg²⁺, Cl⁻, SO₄²⁻, and HPO₄²⁻ (25). The manufacturer claims that Perfadex® "may also be used as a base or 'carrier' solution for other organ-specific electrolytes or active components such as scavengers" (25). Thus, cell morphology can be evaluated with and without antioxidant additives. A functional assessment of lung cells and bronchial epithelium viability in the modified preservative can be performed in mice, and eventually human, lungs. The scope of this study can also be expanded to other transplant organs with a short transport life, such as the heart.

Methods

Antioxidant Preservative Solutions

The following preservatives were characterized: Ice, butylated hydroxytoluene (BHT), vitamin E, vitamin A, vitamin C, lutein, Saffola oil®, melatonin, and biotin. The underlying solvents, distilled water and Saffola oil®, were treated as controls to observe the effects of the water- or fat-soluble additives. BHT, vitamin E, vitamin A, and lutein are fat-soluble; vitamin C, melatonin, and biotin are water-soluble. Ice, the current method

of preservation, was used as the overall reference to compare the effectiveness of antioxidant treatments to the current industry standard. Figure 2 displays the concentrations of preservatives, which were determined based on the maximum daily dose recommended by the manufacturer. All antioxidants were of the same brand (Pharmassure®) except BHT, which was obtained from its manufacturer, Eastman Tenox®; this ensured consistency of excipients.

Preservative solutions were prepared under sterile conditions. After 500 mL of solvent was poured into the sterile container, the corresponding amount of solute was added. The solution was mixed until the solute was evenly distributed in the solvent. All preservative solutions were stored at 4°C.

Tissue Procurement and Preservation

A whole, intact bovine lung was obtained from the San Jose Valley Veal, Inc. It was preserved in the lab in a sterile environment maintained at 3.8–4.4°C. All the results are based off of tissue samples from this single specimen. 6 tissue samples in 2 cm x 1 cm x 1 cm blocks were sliced and used for testing each preservative. Three of these samples were immersed in 250 mL preservative solution for 3 hours, and the other three samples were immersed in solution for 5 hours.

Fixation and H&E Cell Staining

Tissue samples were taken from the preservative solutions and fixed in 10% Neutral Buffered Formalin (NBF) for 48 hours. Then, samples were embedded in paraffin wax (melted on a hot plate at 50°C), processed using a microtome, and stained with H&E. H&E cell staining was used to evaluate the histopathology of epithelial tissue before and after preservation.

Cell Health Evaluation Criteria

In each representative sample slide of the preservative, five fields of view were randomly chosen for evaluation. Sample slides were labeled with letters, and the evaluator was blinded to the particular preservative used on the sample during microscopic evaluation. This was repeated at two time points, 3 and 5 hours. Slides were analyzed for cell damage under a 40x light microscope and 400x microscope, and the field of view was 4500 microns and 450 microns, respectively. Coagulative necrosis, or cell damage, was recorded in both the nucleus and cytoplasm.

Nucleus: Hematoxylin stains nuclei, which are rich in ribonucleic protein, dark blue. Coagulative necrosis is marked by fragmented or missing nuclei.

Cytoplasm: Eosin stains the cytoplasm. Lavender indicates healthy tissue with eosinophilic structures intact, while pink indicates damaged morphology due to excessive permeability through the nuclear envelope.

Rating System

Nucleus and cytoplasm health were each rated on a scale of 1 to 10 (1 = greatest cell damage, 10 = least cell damage). For each sample, the final rating (out of 20) was a sum of these two scores.

A single person evaluated all the slides. A single 40x light microscope was used to evaluate 3-hour samples under the same surrounding light conditions. A

single 400x microscope was used for 5-hour samples. Thus, any random error associated with qualitative measurements, such as color perception, was mitigated.

Acknowledgments

The authors would like to thank Dr. Ting-Ting Huang (Stanford University School of Medicine) for allowing use of equipment, Dr. James Collinson (Quito Vet) and Dr. David Gardiner (IDEXX Laboratories) for help with H&E staining, and Dr. Suparna Dutt (Stanford University School of Medicine) for critical comments on the manuscript.

References

1. "How Many People Get Organ Transplants Every Year?" TransWeb. Ed. Ted Hanss. University of Michigan Transplant Center, 4 Feb. 2010. Web. 10 Sept. 2014.
2. "The Need Is Real: Data." Organdonor. Ed. Sylvia Mathews Burwell. U.S. Department of Health & Human Services, 4 Feb. 2010. Web. 10 Sept. 2014.
3. Nolan, Heather et al. "Artificial lung basics." *Organogenesis*, 2011 Jan-Mar; 7(1): 23-27. Web. 10 Sept 2014.
4. "Lung transplantation" MedlinePlus, National Institutes of Health. 13 August 2014. Web. 10 Sept 2014.
5. "What is a lung transplant?" Health information for the public, National heart, lung and blood institute. 01 May 2011. Web. 10 Sept 2014.
6. Gottlieb, Scott. "Survival after Partial Lung Transplants Is as Good as after Full Lung Transplants." *BMJ* : British Medical Journal. BMJ Publishing Group Ltd., 14 June 2014. Web. 10 Sept. 2014.
7. "Lung Transplant." A.D.A.M. Medical Encyclopedia. Ed. A.D.A.M. Editorial Board. U.S. National Library of Medicine, 6 Jun. 2013. Web. 10 Sept. 2014.
8. Wigfield, C. H. et al. "Successful Emergent Lung Transplantation After Remote Ex Vivo Perfusion Optimization and Transportation of Donor Lungs." Wiley Online Library. *American Journal of Transplantation*, 25 Sept. 2012. Web. 10 Sept. 2014.
9. "How are donated organs preserved and transported?" Transweb – A resource on transplantation and donation. U-M Medical School, 04 Feb 2010. Web. 10 Sept 2014.
10. Cypel, Marcelo et al. "Lung transplantation: Donor lung preservation" UpToDate. Wolters Kluwer Health. 23 Apr 2014. Web. 10 Sept 2014.
11. Oto T, Griffiths A. P., Rosenfeldt F., Levvey B. J., Williams T. J., Snell G. I. Early outcomes comparing Perfadex, Euro-Collins, and Papworth solutions in lung transplantation. *Ann Thorac Surg*. 2006 Nov; 82(5):1842-8. Print.
12. "Penn medicine surgeons perform first ex vivo lung perfusion transplantation in the Philadelphia region" News and Publications. Penn Medicine, 06 Dec 2012. Web. 10 Sept 2014.
13. Bresticker, M. A. et al. "Successful Extended Lung Preservation with UW Solution." National Center for Biotechnology Information. U.S. National Library of Medicine, Nov. 1992. Web. 10 Sept. 2014.
14. Inder, T. E. "Lipid Peroxidation as a Measure of Oxygen Free Radical Damage in the Very Low Birthweight Infant." National Center for Biotechnology Information. U.S. National Library of Medicine, Mar. 1994. Web. 10 Sept. 2014.
15. Madill, J. "Lung Transplantation: Does Oxidative Stress Contribute to the Development of Bronchiolitis Obliterans Syndrome?" National Center for Biotechnology Information. U.S. National Library of Medicine, 23 Apr. 2009. Web. 10 Sept. 2014.
16. Blunhardt, G., and P. Neuhaus. "Does Antioxidant Treatment Have a Role in Liver Transplantation." National Center for Biotechnology Information. U.S. National Library of Medicine, 15 Dec. 1991. Web. 7 Nov. 2014.
17. Weber, Jean-Christophe, Giuseppe Navarra, Long R. Jiao, Joanna P. Nicholls, Steen Lindkaer Jensen, and Nagy A. Habib. "New Technique for Liver Resection Using Heat Coagulative Necrosis." *Annals of Surgery*. U.S. National Library of Medicine, Nov. 2002. Web. 10 Sept. 2014.
18. "Antioxidants: MedlinePlus." U.S National Library of Medicine. U.S. National Library of Medicine, 20 Feb. 2014. Web. 10 Sept. 2014.
19. Helmenstine, Ph.D. Anne Marie. "Chemistry of BHA and BHT Food Preservatives." About Education, 30 May 2014. Web. 10 Sept. 2014.
20. "Vitamin A." A.D.A.M. Medical Encyclopedia. Ed. A.D.A.M. Editorial Board. U.S. National Library of Medicine, 18 Feb. 2013. Web. 10 Sept. 2014.
21. Coulter, I. D., M. L. Hardy, S. C. Morton, L. G. Hilton, W. Tu, D. Valentine, and P. G. Shekelle. "Antioxidants Vitamin C and Vitamin E for the Prevention and Treatment of Cancer." National Center for Biotechnology Information. U.S. National Library of Medicine, n.d. Web. 10 Sept. 2014.
22. Selliah, S., T. De Mel, CA Hewamadduma, and A. De Tissera. "Protective Effective of Vitamin E in Kidney Storage Solution on Renal Tissue Metabolism in Rats." National Center for Biotechnology Information. U.S. National Library of Medicine, Sept. 1995. Web. 10 Sept. 2014.
23. Day, Brian J. "Antioxidants as Potential Therapeutics for Lung Fibrosis." National Center for Biotechnology Information. U.S. National Library of Medicine, Feb. 2008. Web. 10 Sept. 2014.
24. Koo, D.H., Welsh, K.I., et al. "Endothelial cell protection against ischemia/reperfusion injury by lecithinized superoxide dismutase." *Kidney International*, Vol 60 (2001), pp. 786-796.
25. "Perfadex®." Organ Preservation. XVIVO Perfusion, n.d. Web. 10 Sept. 2014.