Ozone Therapy Protects Against Rejection in a Lung Transplantation Model: A New Treatment?

Norberto Santana-Rodríguez, MD, PhD, Pedro Llontop, PhD, Bernardino Clavo, MD, PhD, María D. Fiuza-Pérez, MD, PhD, Keila Zerecero, MVD, Adil Ayub, MD, Khalid Alshehri, MD, Nagib A. Yordi, MD, PhD, Lamberto Re, MD, PhD, Wissam Raad, MD, Leandro Fernández-Pérez, MD, PhD, Ricardo García-Herrera, PhD, Chyun-Yin J. Huang, MD, and Faiz Y. Bhora, MD, FACS

Department of Thoracic Surgery, Mount Sinai Health System, New York, New York; Instituto Universitario de Investigaciones Biomédicas y Sanitarias (IUIBS)-BioPharm Group, ULPGC, Spain; Experimental Surgery, Research Unit, Hospital Dr. Negrín, Las Palmas de Gran Canaria, Spain; Department of Orthopedic Surgery and Upper Extremity Unit, Healthpoint Hospital, Abu Dhabi, United Arab Emirates; Department of Clinical Pharmacology and Toxicology, Medinat SAS, Ancona, Italy; and Academic Division of Agricultural Sciences, Universidad Juárez Autónoma, Tabasco, México

Background. **No satisfactory treatment exists for chronic rejection (CR) after lung transplantation (LT). Our objective was to assess whether ozone (O_3) treatment could ameliorate CR.**

Methods. **Male Sprague-Dawley inbred rats (n = 36) were randomly assigned into four groups: (1) control (n = 6), (2) sham (n = 6), (3) LT (n = 12), and (4) O_3-LT (n = 12). Animals underwent left LT. O_3 was rectally administered daily for 2 weeks before LT (from 20 to 50 μg) and 3 times/wk (50 μg/dose) up to 3 months. CR; acute rejection; and *Hspb27*, *Prdx*, *Epas1*, *Gpx3*, *Vegfa*, *Sftpa1*, *Sftpb*, *Plvap*, *Klf2*, *Cldn5*, *Thbd*, *Dsip*, *Fmo2*, and *Sepp1* mRNA gene expression were determined.**

Results. **Severe CR was observed in all animals of LT group, but none of the O_3-LT animals showed signs of CR, just a mild acute rejection was observed in 1 animal. A significant decrease of *Hspb27*, *Prdx*, *Epas1*, *Gpx3*, *Vegfa*, *Sftpa1*, *Sftpb*, *Plvap*, *Klf2*, *Cldn5*, *Thbd*, *Dsip*, and *Fmo2* gene expression in the O_3-LT group was observed**

Conclusions. **O_3 therapy significantly delayed the onset of CR regulating the expression of genes involved in its pathogenesis. No known immunosuppressive therapy has been capable of achieving similar results. From a translational point of view, O_3 therapy could become a new adjuvant treatment for CR in patients undergoing LT.**

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Lung transplantation (LT) is an effective and applicable treatment in the final stage of non-malignant lung disease. The procedure has developed rapidly since its inception in the 1980s, and clinical outcomes are continually improving. However, long-term outcomes are compromised by chronic rejection (CR) and immunosuppression [1, 2]. Even though more than 47,000 LTs have been performed worldwide, the 5-year survival rate has remained stagnant at approximately 50% since the 1990s [1, 3]. CR is a fibroproliferative process resulting in progressive and irreversible airflow obstruction that is responsible for low long-term survival and is the leading cause of death. The incidence of CR is usually high in the first 2 years after LT, and nearly 50% of the recipients will experience CR by 5 years after LT, with a median survival of 3 years. Even after that, patients remain at undefined cumulative risk between 5 and 10 years after LT. Currently, CR is difficult to detect in the preclinical stage, and few satisfactory treatments exist [4, 5].

Oxidative stress (OS) and the defenses against it are present and genetically regulated in CR [6]. Changes in gene expression through regulation of the transcription factors [7, 8] are essential components of the mechanism that determine the cellular response to oxidative damage [9, 10]. Ozone (O_3)-like molecule is generated in the body during regulation of the immune system [11]. However, by using appropriate dosage and administration route, O_3 can induce a temporary and controlled OS that finally enhances antioxidant defense mechanisms and modulates the immune system [12, 13]. This in turn leads to less

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Address correspondence to Dr Santana-Rodríguez, Department of Thoracic Surgery, Mount Sinai Health System, 1000 Tenth Ave, Ste 2B-07, New York, NY 10019; email: norbesanrod@gmail.com.

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tissue damage as observed in patients with pulmonary [14] or cardiac [15] disease and in experimental animal studies in liver [16, 17], kidney [18], and lung [19]. In addition, O_3 stimulates the release of nitric oxide and prostaglandins in vascular endothelium [12, 13, 18, 20] and increases oxygen delivery to tissues [21, 22]. From these findings, our objective was to assess whether O_3 treatment could ameliorate CR in LT.

Material and Methods

Design of the Study

This study was approved by the institutional animal care committee and followed the Guide for the Care and Use of Laboratory Animals. A total of 36 Sprague-Dawley male inbred rats (300 to 400 g) were used under optimal conditions and randomly assigned into four groups: (1) the control group (n = 6) underwent left thoracotomy, and samples were taken immediately from the healthy left lung; (2) the sham group (n = 6) underwent left thoracotomy without LT; (3) the LT group (n = 12, 6 donors and 6 receptors) underwent unilateral left LT technique with cuffs [23]; and (4) the O_3-LT group (n = 12, 6 donors and 6 receptors) underwent O_3 treatment and unilateral left LT with the same technique. All animals were treated with 0.025 mg/kg/12 h buprenorphine (Buprex; Schering Plug, Barcelona, Spain) subcutaneously during the first 72 hours. No immunosuppressive drugs or antibiotics were administered to prevent the masking of gene expression involved in CR.

O_3 Treatment

A medical O_3 generator (Ozonosan Alpha plus®; Dr. Hänsler Nordring, Germany) was used, controlling the mixed gas concentration (O_3/O_2) with an ultraviolet spectrophotometer at 254 nm. Recipient animals were preconditioned daily with O_3/O_2 10 days before transplantation at increasing doses from 20 to 50 μg/kg by rectal administration in a volume of 20 mL/kg, to assist tolerance to the initial induction of OS. After LT, O_3/O_2 was administered at 50 μg/kg (O_3 total dose of 1000 μg/kg) every 72 hours until the sacrifice.

Postoperative Outcome

Tachypnea, stridor, hemoptysis, and abnormal postures were evaluated during the postoperative period, considering the time and possible cause of death.

Histologic Determinations

Euthanasia of sham and animals that received a transplant was performed 3 months after LT. Rat lung isografts were excised for histologic and molecular evaluation. LT specimens were serially sliced into 10-μm-thick sections and stained with hematoxylin and eosin. An expert LT pathologist (T.M.), blinded to the experimental history of the specimens, performed the pathologic studies. Acute rejection (AR) was evaluated according to the international classification [24]. CR was evaluated according to the presence of signs of CR, such as intimal hyperplasia, periarteriolar fibrosis, interstitial fibrosis, peribroncholar fibrosis, and bronchiectasis and was classified as mild, moderate, or severe [6].

Gene Determinations

Total RNA was isolated by homogenization of frozen tissue using a homogenizer (Polytron PT-2000; Kinematica AG, Luzern, Switzerland) and TriReagent (Sigma, Madrid, Spain), according to the manufacturer's protocol. All samples were treated with RNase-free DNase (Promega, Madison, WI). RNA yields were measured by ultraviolet absorbance, and the quality of total RNA was analyzed with a Bioanalyzer (Model 2100; Agilent Technologies, Palo Alto, CA). The mRNA expression levels of the possible genes involved were measured using quantitative polymerase chain reaction (qPCR) to explore the molecular actions of O_3 treatment (Supplemental Table 1). For qPCR, 1 μg of total RNA was treated with RNase-free DNase I (Promega) to remove genomic DNA and was reverse-transcribed using a reverse transcriptase kit (iScript; Bio-Rad Laboratories, Liège, Belgium). Two microliters of cDNA served as a template in a 20-μL qPCR reaction mix containing the primers and SYBR Green PCR Master Mix (Diagenode, Liege, Belgium). Quantification of gene expression was performed on a sequence-detection system (ABI-PRISM 7000 SD RT-PCR; Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. A dissociation protocol was done to assess the specificity of the primers and the uniformity of the PCR-generated products. The amplified PCR products were subjected to electrophoresis on a 1.5% agarose gel to confirm predicted sizes. Data were extracted, and amplification plots were generated using ABI SDS software. Exon-specific primers were designed by the PRIMER 3 program (Supplemental Table 1) [25]. The level of individual mRNA measured by qPCR was normalized to the level of the housekeeping genes cyclophilin and ribosomal 28S by using the Pfaffl method [26]. For graphing purposes, the relative expression levels were scaled such that the expression of the healthy lung control group equaled one.

Statistical Analyses

Statistical evaluation of histologic findings was performed after samples were graded using standardized nomenclature. Comparisons among the categorical variables were assessed with the χ^2 test. Differences in gene expression activity by qPCR were tested using the Mann–Whitney *U* test. Data management and statistical analyses were performed using SPSS (version 15.0; SPSS Inc, Chicago, IL) statistical software. The value of p less than 0.05 was considered statistically significant.

Results

Postoperative Outcome

All operated animals had a good postoperative outcome, except 2 animals of the LT group that presented with signs of respiratory failure during the first 24 hours. All animals survived the 3-month period up to the sacrifice.