Experimental Lung Transplantation Related With HIF-1, VEGF, ROS. Assessment of HIF-1α, VEGF, and Reactive Oxygen Species After Competitive Blockade of Chetomin for Lung Transplantation in Rats

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Summary

Primary graft failure occurs 15 to 30 % of the time after transplantation. Although there have been improvements in preserving the lungs in good condition, there have not been studies on the regulation of transcription factors. Methods: We carried out an experimental study involving lung transplantation to indirectly evaluate reactive oxygen species (ROS) production and VEGF expression by competitive blockade of HIF-1α with chetomin. There were 5 groups: Group-1: Lung blocks were perfused with 0.9 % SSF, immediately harvested, and preserved. Group-2 (I-T): Immediate transplantation and then reperfusion for 1 h. Group-3 (I-R): Lung blocks were harvested and preserved in LPD solution for 6 h and reperfused for 1 h. Group-4 (DMSO): Lung blocks were treated for 4 h with DMSO, preserved for 6 h and transplanted to a receptor treated with DMSO. Group-5 (chetomin): Lung blocks were treated for 4 h with chetomin, preserved for 6 h and transplanted to a receptor treated with chetomin. ROS, mRNA, and protein levels of HIF-1α and EG-VEGF were determined. Results: The DMSO and chetomin groups had significantly lower ROS levels. Compared with those in the I-R group, the chetomin group exhibited the lowest level of HIF-1α. Conclusions: Addition of chetomin to the donor and the receptor results in a significant reduction in HIF-1A, VEGF and ROS.

Key words

Chetomin • Ischemia-reperfusion • ROS • Lung-Transplantation and HIF1α

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Introduction

Lung ischemia-reperfusion injury is undoubtedly the primary cause of early graft failure, which develops within the first 72 h and represents 15 to 30 % of transplantation adverse events, resulting in early morbidity and mortality [1]. Ischemia causes a series of cascading pathological changes that decrease the activity of life-sustaining systems, resulting in energy depletion.

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One of the main agents of ischemia-reperfusion damage is reactive oxygen species (ROS), which are produced by diverse pathways, such as xanthine dehydrogenase, endothelial stress, and neutrophil-associated stress pathways.

During ischemia-reperfusion, ATP depletion increases the conversion of xanthine dehydrogenase to xanthine oxidase, which produces H_2O_2. In the resting state, xanthine oxidoreductase is present as xanthine dehydrogenase and uses $NADH^+$. During ischemia-reperfusion, there is an increase in reactive oxygen species, with NADPH being the main source of the response to physiological stimuli. NADPH oxidase (NOX) is associated with polymorphonuclear neutrophils (PMNs) because it is the enzyme responsible for respiratory bursts; this mechanism is the source of oxidants for the bactericidal activity of PMNs. In ischemia-reperfusion, endothelial stress favors the expression of NADPH oxidase, which is an important source of ROS in the endothelium [2-4].

This is a consequence of ischemia-reperfusion injury, which is characterized by hypoxia inducible factor (HIF) overexpression and an inflammatory response, leading to edema formation and consequently tissue failure [5,6]. However, decreases in cellular metabolism caused by modern preservation methods (preservation solutions, hypothermia, free radical scavengers, etc.) and the regulation of the expression of transcription factors have not been thoroughly studied [7,8]. Hypoxia inducible factor (HIF-1α) is a heterodimeric complex that is bound to an oxygen-independent β subunit. Under normoxic conditions, HIF-1α is targeted for degradation by prolyl hydroxylase domain (PHD) enzymes and by factor inhibiting HIF (FIH), which hydroxylates specific prolyl residues, resulting in HIF degradation *via* von Hippel-Lindau polyubiquitination, a proteasome-mediated pathway [9]. These latter proteins form part of the ubiquitin E3 ligase complex, causing its correct ubiquitination and degradation; therefore, during normoxia, the HIF-1α protein is expressed but unstable [7,10].

Nevertheless, under hypoxic conditions, the activity of PHD decreases, as does its hydroxylation. HIF-1α is phosphorylated and dimerized with HIF-1β and translocated to the nucleus, where it binds to its CBP/p300 coactivator [11,12]. This results in transactivation and induction of target transcription factors such as vascular endothelial growth factor (VEGF), which affect the permeability of the alveolocapillary endothelium [8,10,13].

Chetomin (dithiodiketopiperacine) is a molecule capable of disrupting the interaction of HIF-1α with the coactivator p300. It binds and interrupts the tertiary structure of the CH1 domain of coactivator p300, preventing the transcription of many genes and thus attenuating hypoxia-induced transcription and its relationship with HIF-1α. In murine experimental models, the use of chetomin to treat tumors inhibits the transcription of HIF-1α [10,14].

The HIF-1α factor is involved in the hypoxia response in some pathologies, such as myocardial ischemia, cerebral ischemia, retinal ischemia, pulmonary hypertension, cardiac bypass surgery, vascular calcifications and tumor processes [12,15]. In experimental lung transplantation, which involves cold preservation for 6 h, HIF-1α has not been inhibited, and orthotopic rat lung transplantation using the cuff technique is an easily reproducible model that can be used to study HIF-1α and VEGF as factors involved in lung ischemia-reperfusion [16,17]. It is clear that an alternative to stabilize the expression of HIF-1α is the use of antitumor drugs, of which chetomin stands out due to its biochemical properties and pharmacological characteristics. Therefore, we assumed that a competitive blockage of cellular metabolism after 6 h of cold preservation of the lung could be indirectly measured by VEGF as a factor dependent on HIF transcription during the first hour of lung transplantation reperfusion, and the aim of this study was to establish a lung transplantation model for studying the genes related to lung ischemia-reperfusion injury.

Materials and Methods

Animals

One hundred twenty-five clinically healthy male Wistar rats (300-320 g) were used and managed according to NOM 062-ZOO-1999 concerning the use and care of laboratory animals; CICUAL-CEX1012-13/16-1, Instituto Nacional de Ciencias Médicas y Nutrición “Salvador Zubirán”.

Validation of lung transplantation surgical technique

Pilot group: (n=40). Twenty lung transplantations were carried out to standardize surgical techniques before the experimental study was performed.

Experimental protocol of study

Experimental study. The rats were divided into

5 groups: Group 1 (n=9): Lung blocks were perfused with 0.9 % SSF, and the left lung was immediately harvested and preserved. Group 2, I-T (n=18): Lung blocks were harvested (n=9) and immediately transplanted into a rat receptor (n=9), and 1 h after reperfusion, the left lung was harvested and preserved. Group 3, I-R (n=18): The lung block was harvested and preserved in LPD solution for 6 h and transplanted and reperfused for 1 h; after that, the left lung was removed and preserved. Group 4, Dimethyl sulfoxide, 5 % (DMSO) (n=18): The lung block was treated with DMSO, which was used as a solvent vehicle for chetomin 4 h prior to its injection, preserved in LPD solution for 6 h, and transplanted into a recipient treated with DMSO (5 %). Group 5, chetomin (dithiodiketopiperacine) 0.5 mg/kg (n=18): Lung blocks were treated with chetomin dissolved in DMSO 4 h before harvesting, preserved for 6 h in LPD solution, and transplanted with a recipient treated with chetomin.

Surgical procedure

Donor

The rats were anesthetized with sodium pentobarbital 50 mg/kg I.P. (Pisa, Hgo. MX), trichotomy was performed in the neck and thoracic area, tracheotomy was performed, and endotracheal catheter #1 (Beckton Dickinson, Brazil) was placed and ventilated (Kent Scientific RSP 1002 ventilator, CT., USA) with a tidal volume of 6 ml/kg and 60-70 breaths/min.

Subsequently, a median sternotomy was carried out. The cranial and caudal cava veins and aortic artery were dissected and ligated (3-0 silk; Johnson and Johnson, Mexico City). Heparin (Inhepar®; Pisa, Mexico City) 100 IU was administered to the right ventricle. The cranial and caudal cava veins and aortic artery were transected. Then, the cardiac apex was sectioned, and through the pulmonary artery (Ap), the lung was perfused with LPD solution (low potassium dextran, designed in the laboratory) at a flow rate of 0.013 ml/kg/min; subsequently, the cardiopulmonary block was harvested [18].

Once the block was obtained, it was wrapped with sterile gauze and placed in a Petri dish, maintaining a hypothermic temperature of 4 °C. The bronchus, artery, and left pulmonary vein were dissected to place a cuff made of an intravenous catheter (16 G for blood vessels and 14 G for the left bronchus) in each of the structures corresponding to the left lung; then, it was preserved for 6 h in LPD solution: NaCl (6.896 g/l), KCl (0.350 g/l), $CaCl_2$ (0.368 g/l), $MgSO_4$ (0.296 g/l), KH_2PO_4 (0.163 g/l), $NaHCO_3$ (2.092 g/l), Glucose (1 g/l), Dextran 40 (20 g/l), Osmolality (300 mOsm/l), pH (7.4).

Recipient

Anesthesia was induced using 50 mg/kg ketamine and 2.5 mg/kg acepromacine intraperitoneally (IP), and meloxicam (5 mg/kg) was subcutaneously injected as an analgesic. It was then intubated in an orotracheal manner by using a 3D mouthpiece [19] with a #16 G catheter connected to a rodent respirator (Kent Scientific RSP 1002, C.T. USA) and kept anesthetized with 2 % isoflurane CAM and 21 % oxygen. A tidal volume of 6 ml/kg, a PEEP of 2-3 cm H_2O, and a 60-70 respiratory rate were applied (BPM).

The recipient was placed in the right lateral decubitus position, and a lateral-left thoracotomy was performed at the level of the 4th intercostal space. The left lung was extracted and placed on the dorsal side to expose the pulmonary hilum; the artery, vein, and bronchus were dissected. The patients were subsequently referred with silk sutures 5-0 (Ethicon, Brazil) in the left pulmonary artery, bronchus, and vein to stop circulation and airflow. Once the tissue was dissected and circulation stopped, a 6-0 prolene (Ethicon, NJ, USA) suture was placed in each blood vessel and bronchus. Subsequently, a V-shaped incision was made proximal to the lung tissue to obtain enough tissue for the maneuvers of the graft. Then, the donor lung artery, bronchus, and vein were introduced in that order and fixed with 5-0 prolene sutures (Ethicon, NJ, USA). The silk sutures were removed to open the circulation and airflow of the bronchus. Then, the lungs were insufflated at normal capacity, and lung reperfusion was carried out for one hour. At the end of the study, a lethal dose of intracardiac pentobarbital was applied for rat euthanasia. Subsequently, left lung biopsies were taken and preserved according to the laboratory molecular or histopathological procedure.

Chetomin dose validation

First, in 4 rats, dose validation was achieved by first administering 0.2 mg/kg chetomin (i.v.) (Enzo, N.J. USA); subsequently, a higher dose (0.5 mg/kg) was used in another 4 rats. Lung graft tissue samples were processed by Western blotting, and the protein synthesis of HIF1-α and EG-VEGF was analyzed. The 0.5 mg/kg chetomin dose did not significantly affect the relative percentage of pulmonary VEGF in pulmonary tissue; the median of 0 mg/kg chetomin (0-0) was significantly different from that of 0.2 mg/kg chetomin, and the median of 0.215 mg/kg chetomin was 0-0.843 (P=0.018), (Fig. 1).

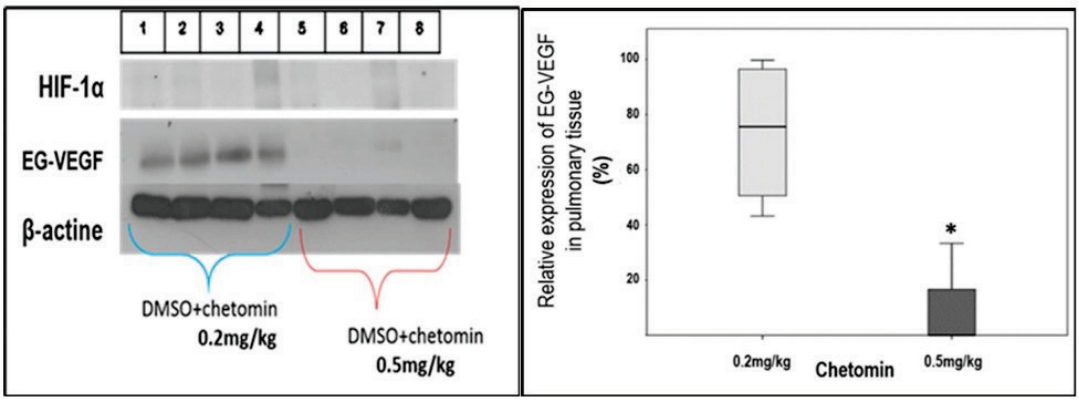

Fig. 1. Two doses of chetomin were used before the experimental study to determine the suitable dose by Western blot analysis and its graphic expressed as the median of EG-VEGF. (**A**) Relative expression of HIF1α, EG-VEGF, and β actin of eighth lung tissue samples. Lines 1-4 dose 0.2 mg/kg and 5-8 dose 0.5 mg/kg. (**B**) Densitometric analyses of EG-VEGF from both chetomin groups. Data are presented as median normalized with β actin as the control protein. * P<0.05 vs. 0.2 mg/kg. U Mann Whitney test.

Protein extraction (determination)/HIF-1α and VEGF Western blot

Pulmonary tissues were stored at -70 °C. Subsequently, the protein levels of HIF-1α and EG-VEGF were determined by Western blotting. First, lung tissue was homogenized and sonicated in buffer containing PBS supplemented with detergent, 0.1 % SDS, 0.5 % sodium deoxycholate and 1 % NP40 and supplemented with the protease inhibitor cocktail Complete® (Roche), 1 mM PMSF, the phosphatase inhibitor NaF (1 mM), and 2 mM sodium orthovanadate. The homogenized tissue was centrifuged at 14000 RPM for 10 min. The supernatants were recovered, and the protein concentration in the solution was quantified by optical densitometry at 595 nm using the Bradford method. The protein samples (120 µg) were separated *via* 10 % SDS-PAGE for HIF-1α or 7.5 % for EG-VEGF and subsequently transferred to PVDF membranes (Merck Millipore). The membranes were incubated with TBS-Tween 0.1 % (TBS-T) with 5 % dehydrated skim milk to block nonspecific protein binding and then incubated with primary antibodies against HIF1-α (1:100 dilution), EG-VEGF (1:100 dilution), and β-actin (1:4000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight. The membranes were washed with TBS-T and then incubated in the presence of HRP-conjugated secondary anti-mouse antibodies (HIF1-α, EG-VEGF diluted 1:5000 and β-actin diluted 1:10000; Pierce Thermo Fisher, Inc., USA) for 60 min at room temperature. Then, the signal was detected using enhanced chemiluminescence solution (Supersignal West Pico Chemiluminescent Substrate; Thermo Fisher Scientific, Inc., USA) with autoradiography film (Juama, Mexico City, MX).

Reverse transcription polymerase chain reaction

Total RNA was obtained from frozen lung tissue using an RNeasy® kit (Qiagen, Germany). The quantity and quality of the RNA were evaluated by spectrophotometry (60/280) with a Nanodrop (Thermo Scientific, USA), and the mRNA was reverse transcribed to obtain cDNA using 50 µg of RNA, Oligo-dt, and an Omniscript reverse transcription kit (Qiagen, Germany). qPCR was carried out to detect the expression of HIF-1α and VEGF, and RT-PCR was subsequently performed with a 7500 Real-Time PCR System (Applied Biosystems, USA) and quantified using Quantitech Sybr Green Master Mix (Qiagen, Germany). The standard curve method was used (1×10^8 to 1×10^2 copies of each gene). Primers were designed with Primer Express software (Applied Biosystems), and the sequences were as follows: Actb; forward, 5' TACTGCCCTGGCTCCTAGCA 3'; reverse, 3′ CTCAGGAGCAATGATCTTGAT 5' (48 fragments). HIF-1α: forward, 5' GTAGTGCTGATCCCTGCACTG-AATC 3'; reverse, 3' CTGGGACTGTTAGGCTCA-GGTG 5' (138 fragment). VEGF: forward, 5' CTTGGG-TGCACTGGACCCT 3'; reverse, 3' CACTTCATGGGC-TTTCTGCTC 5' (100 size fragment).

ROS determination

Reactive oxygen species (ROS) determination was carried out using a thiobarbituric acid reactive substance assay [20]. Lung tissue was homogenized entirely with saline solution; subsequently, the protein concentration was determined by means of the Bradford method. In collection tubes, homogenized tissue (100 µl) was mixed with thiobarbituric acid (0.8 %; 1 ml) and acetate (20 %; 2 ml). The tubes were incubated in water (90 °C for 60 min). Afterward, they were cooled (4 °C).

Then, n-butanol (5 ml) was added, and the tubes were shaken (12 s) and centrifuged (4000 rpm for 10 min). The standard curve was made with 1,1,3,3-tetrahydroxipropane (1-8 nmol). The supernatant was assayed for thiobarbituric acid-reactive substances using a spectrophotometer by measuring the change in the absorbance at 532 nm (Genesis VIS; Thermo Scientific), and the level of malondialdehyde (MDA; nmol) was reported per mg of tissue protein.

Statistical analysis

For dose validation of chetomin, the data are expressed as the median, and the Mann-Whitney U test was performed with a $P<0.05$ indicating statistical significance. The data are expressed as the mean ± standard error. The statistical significance of differences between the groups was determined by one-way analysis of variance (ANOVA) and *post hoc* Tukey's test. A $P<0.05$ denoted statistical significance. SPSS statistical package version 25, IBM corporation, NY, USA, was used for all the statistical analyses.

Results

Pilot group

The pilot group was subjected to standardization of the surgical procedure and management of that variable before beginning the experimental study. After 20 orthotopic transplants of the left lung were performed *via* the cuff technique (Fig. 2). The total duration of the experiment of lung transplantation were the following: Procurement: 0:51±0:06. Transplantation: 0:50±0:04. Total time of surgical procedure: 1:41±0:07. The values were expressed in h:min ± standard error.

Reactive oxygen species

ROS levels were measured by MDA formation. Compared with those in the control, DMSO, and chetomin groups, the ROS levels in the I-R group were significantly greater. The immediate-transplantation group showed the second-highest ROS production, and the values were significantly different from those of the chetomin, ischemia/reperfusion, and control groups (Fig. 3).

mRNA of HIF and VEGF

After 6 h of ischemia and 1 h of reperfusion, the lung-transplanted tissues of the I-R group had the highest levels of mRNa expression of HIF-1α, followed by those of the DMSO group and I-T group. Nevertheless, compared with those in the I-R group, the levels of HIF-1α in the chetomin group were significantly lower;

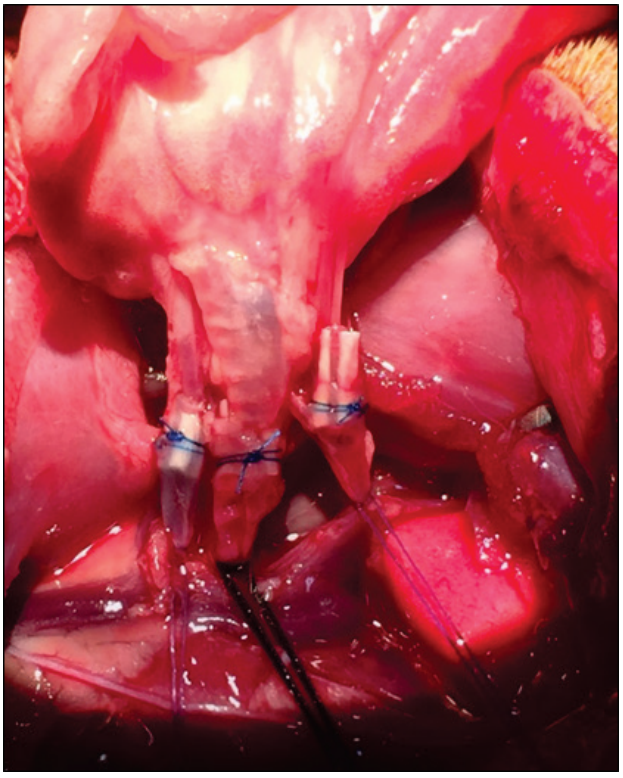

Fig. 2. The image shows the locations of the cuffs and vascular anastomosis during lung transplantation in Wistar rats.

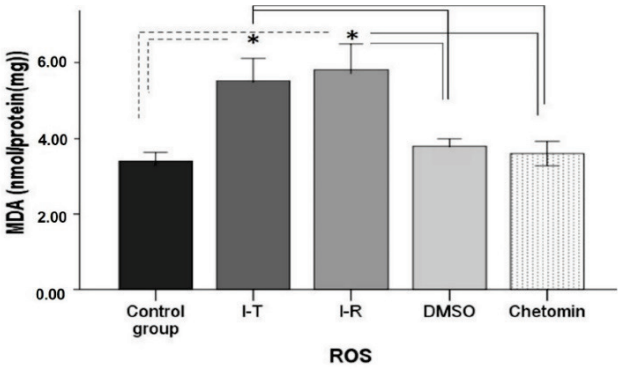

Fig. 3. The ROS concentration was determined by MDA quantification. The data are expressed as the means ± S.E. ANOVA with *post hoc* Tukey test. $P<0.05$.

therefore, a decrease in the nuclear level of the p300 cofactor, which is the site of HIF-1α translocation and dimerization with HIF-1β, was specifically observed Fig. 4). VEGF was evaluated as a dependent factor of HIF-1α, and mRNA expression of VEGF in lung tissues showed that the chetomin group had the lowest levels of VEGF in comparison with the other study groups (Fig. 5).

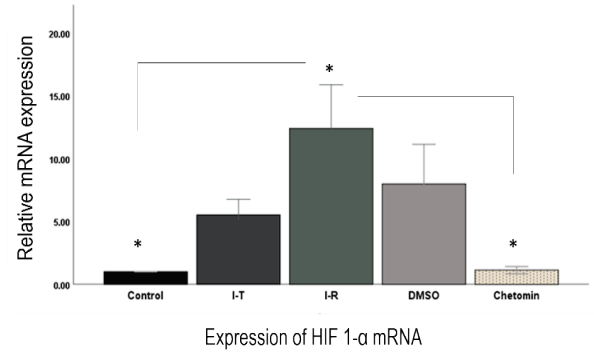

Fig. 4. mRNA expression of HIF-1α after lung transplantation. The data are expressed as the mean ± S.E. ANOVA with *post hoc* Tukey's test. P<0.05.

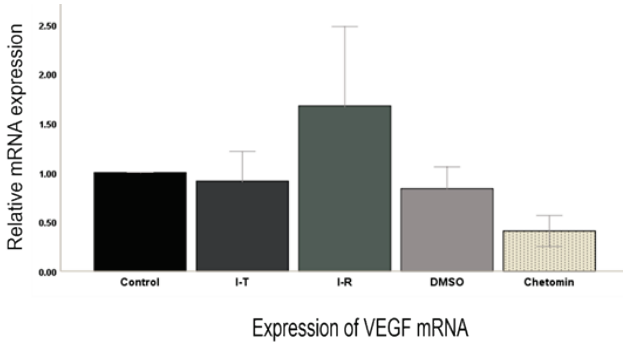

Fig. 5. mRNA expression of VEGF after lung transplantation. The data are expressed as the mean ± S.E. ANOVA with *post hoc* Tukey's test. P<0.05.

Discussion

In this work, a competitive blockade of HIF-1α was performed in an ischemia-reperfusion model to indirectly assess the effect of this factor on VEGF by administering chetomin. The rat pulmonary transplant model has been used to study various mechanisms related to the damage that occurs in the first 72 h and leads to early graft failure. One of these factors is the increase in vascular permeability, which triggers the formation of edema. This is strongly related to VEGF because this factor is involved in vascular permeability during the first hours of VEGF overexpression.

Lung ischemia-reperfusion injury is the first cause of primary graft dysfunction after lung transplantation; this phenomenon appears in the first 72 h of transplantation, leading to pulmonary edema and tissue failure. We established orthotopic lung transplantation using the cuff technique; after the pilot study, we observed that the transplantation time was similar to that reported by other authors.

It then designed an experimental study with five groups. Although primarily driven by the T-cell response, the role of complement activation in acute graft rejection has become evident in recent years, and hyperacute graft rejection is rare. It may occur immediately after allotransplantation due to preexisting blood group-directed or anti-MHC antibodies. The binding of preexisting, so-called natural antibodies, mostly of the IgM class, to donor cells leads to fulminant activation of the complement and clotting systems with rapid loss of graft function. This was not the case in the present work. Additionally, the rat MHC is considerably less polymorphic than that of other mammals, such as mice, primates and humans. Therefore, the experiments presented here do not show strong immune responses during the early stages of transplantation. However, whether graft rejection will occur in the following stages remains to be determined.

A chetomin dose of 0.5 mg four hours before the surgical procedure was standardized by Western blotting, and we measured the relative percentage of EG-VEGF (Fig. 6). Thus, chetomin bound to p300 and inhibited HIF-1α nuclear translocation, disrupted HIF-1α transcriptional activity, and did not affect VEGF protein expression.

After six hours of ischemia and one-hour of transplantation, we found that the levels of ROS related to the ischemia-reperfusion period were decreased by administering chetomin. This drug strongly affected ROS formation after 6 h of ischemia and 1 h of reperfusion in our lung transplantation experimental model. We performed a 1-hour test on the basis that we measured the production of mRNA to study the effect of chetomin on VEGF expression, as there is no other work testing this novel experimental therapy.

HIF-1 is a master regulatory key that maintains cell and systemic homeostasis in response to hypoxic conditions and is regulated by the alpha subunit (HIF-1α), which is stabilized during any ischemic process that leads to cellular hypoxia [25]. Such as lung transplantation, among any other pathologies.

To date, no studies have shown a decrease in the formation of oxygen free radicals in animal models of lung transplantation after 6 h of cold preservation. Guerra *et al.* found that at 6 h after ischemia and reperfusion in an ex vivo model by using an isolated reperfusion system, ROS were decreased through the use of vasodilators (sildenafil); however, this research does not involve lung transplantation [20].

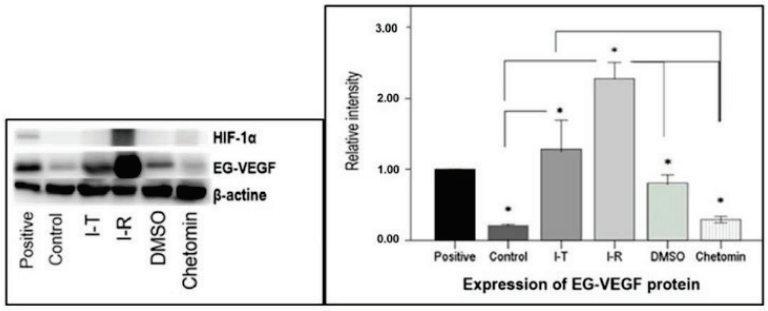

Fig. 6. (**A**) Western blot analysis of HIF-1α, EG-VEGF and β-actin protein expression. (**B**) Relative intensity of EG-VEGF protein; the data are expressed as the mean ± S.E. ANOVA with *post hoc* Tukey test. P<0.05.

When chetomin is administered, HIF1-α is competitively blocked because the P300/CBP binding site does not allow dimerized HIF to bind during nuclear translocation. Consequently, there is no transcription for VEGF-expressing genes or other genes related to cell metabolism in a reduced oxygen environment, such as the GLUT-1 gene and its activity with NADPH oxidase related to the formation of oxygen free radicals, one of which is hydrogen peroxide (H_2O_2), and at the time of reperfusion, it also begins to generate peroxynitrites due to the presence of oxygen when airflow is restored. Zepeda *et al.* reported that there may be a close interaction between HIF1-α and ROS formation related to NADPH oxidase and mitochondrial electron transport, although this interaction is still being explored [10]. Another study by Balogh *et al.* in a hypoxia-induced vascular calcification model evaluated the formation of reactive oxygen species in lung tissue by administering chetomin (6 ml/l) and observing a decrease in ROS, suggesting an interaction between HIF-1α and ROS [21,22]. In another study by Shampa *et al.*, the interaction between HIF-1α and ROS was demonstrated by the overexpression of $P22^{phox}$, which is a subunit of NADPH that leads to an increase in HIF-1α in smooth muscle cells [2].

Thus, corresponding value is found in the comprehension of acute and chronic rejection of the allograft. By definition, every transplant performed corresponds to a period of ischemia and reperfusion, and to date, death occurs in 10-20 % of patients in the first year, and 59 % of patients at 5 years after transplantation [23,24] is attributable to chronic lung allograft dysfunction (CLAD) in 50 % of cases. In each of the possibilities causing CLAD (discarding infection), an inflammatory response whose cornerstone is the initial formation of ROS can be studied [25]. It is well known that exaggerated production of ROS also occurs in multiple pathologies that lead to lung transplantation, such as COPD [26], or an organ failure that requires temporal substitution, such as ARDS [27]. The blockade of HIF-1A suggests a new possibility for understanding lung injury related to ROS; as we can see in this model, there is a significant reduction in ROS, which can translate to a potentially better outcome, assuming that the inflammatory response is directly related to ROS production. There are a few limitations to this study. The first is the short survival time, and the second is the study of only 1 metabolic trait involved in ROS formation (HIF-1A, VEGF) [28,29].

Conclusions

Adding a 0.5 mg/kg dose of chetomin to the donor and the receptor before and after transplantation significantly reduced HIF-1A and VEGF expression and ROS production. The interaction between HIF and ROS plays an important role in ischemia-reperfusion injury after lung transplantation, at least in experimental research. The inhibition of HIF-1 could regulate ROS formation at the NADPH level oxidase. Further investigations are required to establish the safety of long-term usage of this molecule and its long-term effects as a potential anti-inflammatory drug. Furthermore, establishing a dose at which HIF-1α is inactivated is highly valuable for establishing new models of I-R that involve 6 h of cold preservation and 1 h of reperfusion.

As an initial study, this work provides interesting insights into the role of HIF-1A blockade in ischemia-reperfusion phenomena related to lung transplantation, but further experiments, including investigations of the temporality of reperfusion, focusing on pulmonary graft function and immunocompatibility, should be performed.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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