Acute exposure to C60 fullerene damages pulmonary mitochondrial function and mechanics

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ABSTRACT
C60 fullerene (C60) nanoparticles, a nanomaterial widely used in technology, can offer risks to humans, overcome biological barriers, and deposit onto the lungs. However, data on its putative pulmonary burden are scanty. Recently, the C60 interaction with mitochondria has been described in vitro and in vivo. We hypothesized that C60 impairs lung mechanics and mitochondrial function. Thirty-five male BALB/c mice were randomly divided into two groups intratracheally instilled with vehicle (0.9% NaCl + 1% Tween 80, CTRL) or C60 (1.0 mg/kg, FUL). Twenty-four hours after exposure, 15 FUL and 8 CTRL mice were anesthetized, paralyzed, and mechanically ventilated for the determination of lung mechanics. After euthanasia, the lungs were removed en bloc at end-expiration for histological processing. Lung tissue elastance and viscosity were augmented in FUL group. Increased inflammatory cell number, alveolar collapse, septal thickening, and pulmonary edema were detected. In other six FUL and six CTRL mice, mitochondria expressed reduction in state 1 respiration [FUL = 3.0 ± 1.14 vs. CTRL = 4.46 ± 0.9 (SEM) nmol O2/min/mg protein, p = 0.0210], ATP production (FUL = 122.6 ± 18 vs. CTRL = 154.5 ± 14 lmol/100 lg protein, p = 0.0340), and higher oxygen consumption in state 4 [FUL = 12.56 ± 0.9 vs. CTRL = 8.26 ± 0.6], generation of reactive oxygen species (FUL = 733.1 ± 169.32 vs. CTRL = 486.39 ± 73.1 nmol/100 lg protein, p = 0.0313) and reason ROS/ATP [FUL = 8.73 ± 2.3 vs. CTRL = 2.99 ± 0.3]. In conclusion, exposure to fullerene C60 impaired pulmonary mechanics and mitochondrial function, increased ROS concentration, and decrease ATP production.

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1. Introduction
Nanomaterials have been widely used in several areas, from technology and industry to medicine (Sanchez and Sobolev 2010). Their physicochemical characteristics triggered their broad use, despite the still little elucidated environmental and human toxicological risks (Buzea, Pacheco, and Robbie 2007; Fresta et al. 2018). Therefore, further investigations on the interactions between these apparently non-cytotoxic nanomaterials and different cell types are needed.

Fullerene C60 is a nanomaterial formed by 60 carbon atoms (Santos et al. 2010; Sumner et al. 2015). Experimentally discovered in 1985, undoubtedly C60 is the most abundant and representative among stable fullerenes so far isolated (Kroto et al. 1985; Goodman, Gershwin, and Bercovich 2012). C60 is also considered as a nanopollutant and its toxicological risks have become an issue owing to its significant release into the atmosphere by anthropogenic sources, such as the combustion of petroleum products (gasoline and diesel), biomass burning, and industrial waste (Baker et al. 2008; Hendren et al. 2011).

Urban atmospheric gas samples containing fullerene suggest its inhalation by humans (Utsunomiya et al. 2002; Baker et al. 2008; Sayers et al. 2016). Studies have already demonstrated the toxicity of
fullerenes and nanoparticles in general. They can overcome biological barriers due to their small size and cross the lung natural barrier, deposit onto the alveolar space, and cause large inflammatory cell infiltrate associated with alveolar interstitial edema and hemorrhagic changes, damage type I epithelial cells, and impair lung mechanics (Inoue et al. 2009; Tang et al. 2013; Arick et al. 2015; Botelho et al. 2016).

Similar to other nanomaterials, fullerene exposure has been strongly related to mitochondrial dysfunction by means of different toxic mechanisms (Freyre-Fonseca et al. 2011; Liu et al. 2019; Santos et al. 2014; Dong et al. 2016; Xu et al. 2016). Mitochondria play an essential, but not unique, role in cellular respiration, where oxygen is consumed and adenosine triphosphate (ATP) is produced during oxidative phosphorylation. Indeed, during this process superoxide anion ($O_2^-$) is also generated and converted into hydrogen peroxide ($H_2O_2$) by manganese superoxide dismutase (MnSOD), which crosses the mitochondrial membrane (Spinelli and Haigis 2018). An impaired oxidative phosphorylation may lead to mitochondrial dysfunction and trigger an oxidative imbalance, which can be used to gauge the toxicity (Moreno et al. 2007; Yang et al. 2016). C60 and derivatives can induce cytotoxicity, trigger apoptosis by the increase in reactive oxygen species (ROS), and reduce mitochondrial membrane potential and capacity (Jacobsen et al. 2008; Lee et al. 2011). However, most studies investigate isolated mitochondrial activity or on the cellular level, with no further detail about the possible change at the organ level, especially those broadly exposed to nanopollutants, as the lung.

Recent data demonstrate an imbalance of energy metabolism and mitochondrial dysfunction resulting from exposure to low doses of nanoparticles, even though they are labeled as nontoxic (Fresta et al. 2018). Considering: (1) the scarcity of reports in the literature dealing with the possible acute toxic actions of fullerene on lung mechanics and the lack of information on low-dose C60 action on mitochondrial function ex vivo postexposure, and (2) that molecular dynamics simulations disclose the stronger fullerene C60 interaction with regions/organelles constituted by lipid bilayers than with any other biological membranes (Santos et al. 2014), we hypothesized that exposure to a low dose of fullerene C60 may impair lung mechanics, inflammatory status in vivo, and mitochondrial function ex vivo. For such purpose, mice were exposed once to fullerene C60.

2. Material and methods

2.1. Animals

Male BALB/c mice (5–8 weeks) from the Animal Facilities, Health Science Center, Federal University of Rio de Janeiro, weighing 25 ± 2 g (mean ± SD) were employed. The animals received care according to the following guidelines: ARRIVE, National Council for Animal Experimentation Control, Ministry of Science, Technology, and Innovation (CONCEA/MCTI), Brazil, the “Principles of Laboratory Animal Care” formulated by the “National Society for Medical Research”, and the “Guiding Principles in the Care and Use of Animals” approved by the Board of the American Physiological Society. The Animal Ethics Committee on the Use of Animals, Health Sciences Center (CEUA/CCS), Federal University of Rio de Janeiro, approved the present study (code: 155/18).

2.2. Experimental design

We used a single instillation of C60 at a dose considered nontoxic (three-times below the estimated LOAEL) (Shinohara, Gamo, and Nakanishi 2011). Thirty-five mice were randomly divided into two groups. Control group (CTRL, $n = 14$) received intratracheally 50 μl of saline solution (0.9% NaCl) with 1.0% sterile polysorbate 80/Tween 80 (Merck, Darmstadt, Germany). Fullerene group (FUL, $n = 21$) was instilled with 50 μl of fullerene C60 in 1.0% Tween 80 (1 mg/kg BW). Shortly thereafter, these groups were subdivided to undergo two experimental protocols: (1) 8 CTRL and 15 FUL mice were assigned to the assessment of lung mechanics and morphology, and (2) 6 animals from each group were allotted to the determination of mitochondrial function. Before instillation, anesthesia was induced and maintained into the anesthetic plane with isoflurane 5 and 3%, respectively, employing an Isotec 3 vaporizer (Ohmeda, BOC Health Care, West Yorkshire, England). The animals were placed on an operating table and underwent a cervical mid-line longitudinal incision of approximately 1 cm to
expose the trachea and to intratracheally instillate vehicle or fullerene. Xylocaine gel 2% was applied to the wound and the incision was closed with 5.0 suture. The mice quickly recovered and were then returned to their cages.

2.3. Fullerene C60

C60 was prepared as previously reported (Park et al. 2010). Considering that C60 fullerenes between 45 and 60 nm in diameter were detected in several sources such as coal, oil refinery, rivers, atmosphere, this study used the same fullerene-C60 with purity greater than 99.5% from Sigma-Aldrich (St. Louis, MO) used in previous studies (Sanchís et al. 2013; Oliveira et al. 2018; Gredilla et al. 2019). Briefly, 16.7 mg of fullerene C60 were suspended (Sigma-Aldrich, St. Louis, MO) and added to 50 mL of toluene (Merck, Damstadt, Germany) under continuous stirring for 2 h at 150 rpm on a magnetic stirrer. The C60 solution in toluene was added to the saline solution (0.9% NaCl with 1.0% polysorbate 80/Tween 80 (Merck, Darmstadt, Germany) yielding a 20-mL total volume. A heated (37°C) ultrasonic bath (Branson® M2800H, Danbury, CT) allowed the complete volatilization of the toluene shortly thereafter. The suspension was passed through a 0.22-μm sterile disposable filter (Merck, Darmstadt, Germany). Samples were stored in aliquots in a freezer (−20°C).

The particles were considered nanosized (Pinheiro et al. 2021). Briefly, the distribution of emulsion particle sizes was determined using the Zetasizer ZS DLS analyzer (Malvern Instruments Ltd., Worcestershire, UK). The frequency distribution of particle diameters ranged from 28.21 to 164.2 nm: 60% ranged between 37.84 and 50.75 nm, with a peak at 48.07 nm, and 1.3% with a diameter above 100 nm.

2.4. Respiratory mechanics

Twenty-four hours after intratracheal vehicle or fullerene instillation, the animals were intraperitoneally sedated and anesthetized with xylazine (10 mg/kg BW) and ketamine (120 mg/kg BW), sufficient to maintain an adequate anesthetic plane (suppression of the corneal-eyelid reflex) for 1 h. After anesthesia, the animals were paralyzed (pancuronium bromide, 0.1 mg/kg, i.v.), connected to a small animal ventilator (flexiVent, SCIREQ, Montreal, QC, Canada) driven by flexiWare software (SCIREQ, Montreal, QC, Canada), and mechanically ventilated (tidal volume: 8 mL/kg, ventilatory rate: 90 breaths per minute, positive end-expiratory pressure (PEEP): 2 cmH2O (Saldiva et al. 1992); and inspiration: expiration ratio equal to 1:2). The anterior chest wall was surgically removed.

The measurement of pulmonary impedance components was based on the constant phase model (Hantos et al. 1992). For this purpose, an 8-s-long forced multifrequency (0.2–20 Hz) oscillation was generated by the aforementioned ventilator. The pressure and flow produced by the oscillations allowed the calculation of pulmonary impedance components: Newtonian resistance ($R_N$), tissue viscosity, and elastance (G and H, respectively), and hysteresis ($\eta$). The iner tance of the system was assumed as negligible in the range of frequencies applied. Eight measurements were run, each one followed by a 20-s interval of regular breathing.

After the determination of pulmonary mechanics, heparin (1000 IU) was intravenously injected and the trachea was clamped at end-expiration, immediately followed by euthanasia by sectioning the abdominal aorta and vena cava, which led to massive hemorrhage. The lungs were then removed en bloc. The experiments lasted about 30 min.

2.5. Histological study

The left lung was fixed at end-expiratory lung volume with Millonig’s formaldehyde, routinely prepared for histology, and embedded in paraffin. Four-mm-thick slices were cut and stained with hematoxylin-eosin. Lung histology was evaluated with an integrating eyepiece with a 100-point, 50-line coherent system coupled to a light microscope (Axioplan, Zeiss, Oberkochen, Germany). The point-counting technique was realized as previously reported (Zin et al. 2012; Mazzoli-Rocha et al. 2014). Briefly, using different magnifications and 10 random noncoincident microscopic fields, points falling on normal alveoli, and collapsed airspaces were counted and divided by the total number of points hitting alveoli in each microscopic field, while points falling on mononuclear (MN) and or polymorphonuclear (PMN) cells were counted and
divided by the total number of points falling on tissue area in each microscopic field (Weibel 1990).

2.6. Mitochondria isolation

For this purpose, euthanasia occurred by cervical dislocation. The lungs were rapidly removed, placed in ice-cold isolation buffer [in mmol/L: 250 sucrose, 10 HEPES, ethylene glycol-bis(β-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid (EGTA), pH 7.4 with 0.5% w/v bovine serum albumin (BSA)], thoroughly minced using scissors, and then homogenized with a tissue homogenizer (IKA Ultra-Turrax T10, Sigma-Aldrich Labware, São Paulo, Brazil) using two 10-s treatments at a shaft rotation rate of 6500 rpm. This homogenate was further homogenized using a Teflon pestle. The homogenate was centrifuged (Mikro 200 R, Hettich, Tuttlingen, Alemanha) at 700 g for 10 min at 4°C. The supernatant was collected and centrifuged at 12,300 g for 10 min. The resulting pellet was re-suspended in isolation buffer without BSA and centrifuged at 10,300 g for 10 min at 4°C. This procedure was repeated, and the pellet was re-suspended in isolation buffer. The protein concentration of the isolated pellet was determined using a protein assay (Lowry method, Bio-Rad, Hercules, CA) by comparison to a BSA standard (Thermo Scientific, Waltham, MA) (Maciel et al. 2020).

2.7. Mitochondrial oxygen consumption

Mitochondrial oxygen consumption was measured as previously reported (Maciel et al. 2020). Briefly, a Clark-type electrode (Strathkelvin, Glasgow, UK) was used at 37°C during magnetic stirring in incubation buffer containing in mmol-L−1: 125 KCl; 10 MOPS; 2 MgCl2; 5 KH2PO4; 0.2 EGTA; 5 pyruvate and 5 malate (as substrates for complex I). Mitochondria corresponding to 300 μg of mitochondrial protein were added to 1 ml incubation buffer and the following parameters of mitochondrial function were evaluated: complex I state 2 respiration (oxygen uptake before the ATP-synthase activation); complex I state 3 respiration rate (oxygen uptake in the presence of ADP (1 mmol-L−1); state 4 respiration rate (oxygen uptake in the presence of oligomycin); complex IV respiration and maximal uncoupled oxygen uptake was measured in the presence N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD, 300 μmol-L−1) plus ascorbate (3 μmol-L−1); and uncoupled respiration was monitored after addition of carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone phenyldrazone (FCCP, 30 nmol-L−1).

2.8. Mitochondrial ATP production, resynthesis, and ROS generation

After the measurement of ADP-stimulated respiration, the incubation buffer containing mitochondria was taken from the respiration chamber and immediately supplemented with ATP assay mix (ATP Bioluminescence Assay Kit, Sigma-Aldrich, St. Louis, MO) diluted 1:5. Mitochondrial ATP production was determined immediately after each respiration measurement and compared with ATP standards using a 96-well white plate and a spectrofluorometer (SpectraMax® M3, Molecular Devices, San Jose, CA) at 560-nm emission (Maciel et al. 2020).

We used the Amplex Red Hydrogen Peroxide Assay (Life Technologies, Carlsbad, CA) to determine the extramitochondrial ROS concentration. Amplex Red reacts in 1:1 stoichiometry with peroxide in the presence of horseradish peroxidase (HRP) and produces highly fluorescent resorufin. The incubation buffer containing mitochondria was removed from the respiration chamber and immediately supplemented with 50 μmol-L−1 Amplex UltraRed Reagent (Thermo Scientific, Waltham, MA) and 2 U/mL HRP (Sigma-Aldrich, St. Louis, MO). After 120 min of incubation in the dark, the supernatant was collected. Extramitochondrial ROS concentration was determined and compared with H2O2 standards at 540-nm emission and 580-nm extinction, as previously reported (Maciel et al. 2020).

The ratio ROS/ATP (H2O2 production/ATP formation), the oxygen consumption associated with ATP resynthesis (complex I state 3 and state 4), and the electron leakage (O2 consumption/H2O2 production) were also analyzed.

2.9. Mitochondrial swelling and transmembrane potential (ΔΨm)

Mitochondrial swelling and transmembrane potential were evaluated using a spectrofluorometer (SpectraMax® M3, Molecular Devices, EUA). The integrity of the mitochondrial membrane was assessed by osmotically induced mitochondrial volume changes and spectrophotometric determination...
of the apparent absorption of the suspension at 540 nm. A mitochondrial suspension (100 µg/ml) was added to the respiration medium in the absence of respiratory substrates, at 37 °C and under constant stirring. The mitochondrial turgor was stimulated with 100 nmol·L⁻¹ calcium. The swelling was expressed as a percentage of the absorption of the solution containing mitochondria in the presence of cyclosporin A (0% of mitochondrial turgor), in relation to that absorbed after the addition of FCCP (100% of mitochondrial turgor). TMRM (tetramethylrhodamine methyl ester, 400 nmol·L⁻¹) was added to the incubation solution containing 100 µg/ml of mitochondria for 1 h for the determination of mitochondrial transmembrane potential (Δψm). Δψm was estimated by fluorescence emitted by TMRM under 580 nm excitation. Δψm was expressed as a percentage of fluorescence emitted by TMRM-labeled mitochondria in the presence of cyclosporin A (0% of mitochondrial depolarization), relative to that emitted after the addition of FCCP to fully depolarize the mitochondria (100% of mitochondrial depolarization).

2.10. Statistics
Statistical analysis used GraphPad Prism 5.00 (San Diego, CA). Initially, the normality of the distributions of respiratory mechanics and histology data were evaluated by the Kolmogorov–Smirnov test with Lilliefors’ correction. Next, the homogeneity of variances was tested (Levene’s median test). If both conditions were met, the two groups were compared using Student’s t-test, with data expressed as mean ± standard error of mean (SEM). If one of them was not satisfied, the nonparametric Mann–Whitney test was used instead, being the data expressed as medians, 25–75% percentiles, and upper and lower limits. Mitochondrial oxygen uptake, ATP production, and ROS data were analyzed by either paired t-test or, if the conditions were not met, by the Wilcoxon nonparametric test. In all instances, differences were considered significant when p < 0.05.

3. Results
3.1. Respiratory mechanics
Newtonian resistance (Rn) and hysteresivity (η) did not differ between groups (Figure 1(A,D), respectively). Conversely, pulmonary tissue resistance and elastance augmented in FUL mice 24 h after instillation of C60 (Figure 1(B,C), respectively).

3.2. Histological inflammatory profile and alveolar collapse
Photomicrographs of lung parenchyma (Figure 2) supports the findings of mechanical impairment and depict inflammatory pattern with alveolar septal thickening, areas of alveolar collapse, inflammatory cell infiltrate, and pulmonary edema in FUL group. The amount of mononuclear cells (MN) in FUL mice did not differ from CTRL (Figure 3(A)). However, the fraction of PMN cells in the lung parenchyma and collapsed alveoli were higher in FUL than in CTRL mice after a single exposure to fullerene C60 (Figure 3(B,C), respectively).

3.3. Mitochondrial oxygen consumption, ATP production, and ROS generation
The basal mitochondrial respiration did not differ between groups (Figure 4(A)). The oxygen consumption of mitochondria isolated with pyruvate/malate for complex I in the state 2 stimulation was reduced in FUL mice compared to CTRL (Figure 4(B)). However, the ADP stimulation was capable to improve the complex I respiration in state 3 of isolated lung mitochondria, indicating no damage to phosphorylative oxidation in both groups (Figure 4(C)). The ATP-synthase inhibitor (mitochondrial state 4) improved FUL mice mitochondrial oxygen consumption, compared to CTRL mice (Figure 4(D)). Mitochondrial complex IV respiration and maximal oxygen uptake of uncoupled mitochondria were not different between groups, reflecting an equal loading of viable mitochondria in all experiments (Figure 4(E)). Mitochondrial ATP production in FUL group was smaller than in CTRL rats (Figure 4(F)). Mitochondrial ROS generation was higher in FUL than in CTRL group (Figure 4(G)). The ratio between ROS formation and ATP resynthesis (Figure 4(I)) and electron leakage (Figure 4(J)) did not differ between groups.
3.4. Mitochondrial transmembrane potential (Δψm) and swelling

Mitochondrial Δψm and swelling did not differ between groups (Figure 4(K,L)). Despite an upward trend in the FUL group, mitochondrial swelling showed the same pattern as mitochondrial transmembrane potential, i.e. no difference between groups (Figure 4(L)).

4. Discussion

The present study aimed to analyze the putative pulmonary mechanics alterations and mitochondrial toxicity after an intratracheally instilled single low dose of fullerene C60. Carbon nanodiamonds in nontoxic concentrations triggered an imbalance of energy metabolism and mitochondrial dysfunction in vitro, stressing the importance of fully assessing biosafety and toxicity also in vivo (Fresta et al. 2018). Although the estimated C60 fullerene no-observe-adverse-effect level (NOAEL) was calculated considering data presented so far (3.0 mg/kg) (Shinohara, Gamo, and Nakanishi 2011), more recent data support our findings and demonstrate that inflammatory responses, pulmonary mechanical impairment, and reproductive dysfunctions can be triggered at 24 h of a single exposure, using a dose about three-times lower (Park et al. 2010; Pinheiro et al. 2021). Therefore, we may be underestimating the toxic potential of nanoparticles, as well as overestimating the acceptable exposure time limit.

The absorption and distribution of fullerene depend largely on the route of exposure. The lung structure is vulnerable to a wide variety of pollutants because of its permanent contact with the external environment and its broad surface area. Hence, pulmonary susceptibility to the action of molecules originating from exogenous sources is commonplace (Kirkham and Barnes 2013; Lao et al. 2014). Due to the complexity of the pulmonary system, and the potential interactions between the fullerene C60 nanoparticle and the pulmonary parenchyma (Seiffert et al. 2015), the latter needs to be further investigated. Additionally, the pulmonary mitochondrial toxicity putatively triggered by C60 nanoparticles has not been so far explored.
Our study showed that animals exposed to C60 fullerene suspension presented impaired lung function, as evaluated by increased pulmonary tissue resistance (G) and elastance (H) 24 h after a single administration (Figure 1). Our group had already analyzed lung mechanics as a function of time after exposure to C60 and found augmented tissue viscosity and elastance, mainly at 24 h (Pinheiro et al. 2021). Our data are also in accordance with another study that used forced oscillations to evaluate lung mechanics in mice intratracheally instilled with low doses of silver nanoparticles (AgNPs) and the same time point as ours (Botelho et al. 2016). These results can be explained anatomically. Although factors such as gravitational sedimentation and inertial impactation interfere in the lung transport of NPs, decreasing particle size enhances deposition, and retention in the distal airways and alveolar regions, jeopardizing pulmonary surfactant function, which undermines the mechanical stability of airspaces (Anjilvel and Asgharian 1995; Arick et al. 2015).

The mechanical impairment coincide with the increase in inflamed areas containing cellular infiltrate, mainly neutrophils, alveolar septal thickening, presence of pulmonary edema, and alveolar collapse. Our group also demonstrated these findings triggered by other micro- and nano-pollutants (Zin et al. 2012; Carvalho et al. 2014; Mazzoli-Rocha et al. 2014). In this line, hemorrhage occurs in the alveoli after a single fullerene instillation (Inoue et al. 2009; Ema et al. 2012). Similarly, electron microscopy evidenced impairment of the alveolar-capillary barrier after intratracheal instillation of ultrafine carbon particles, resulting in a large infiltrate of inflammatory cells associated with edema, alveolar interstitial injury, and damage to type I epithelial cells (Inoue et al. 2009). Ultrastructural changes were also disclosed in mice after intratracheal instillation of Asian sand dust and gold nanoparticles 24 h after exposure (Ema et al. 2012; Rattanapinyopituk et al. 2013).

Figure 2. Photomicrographs of pulmonary parenchyma (upper panels, 100×; lower panels, 400×) stained with hematoxylin–eosin. CTRL, animals were intratracheally instilled with saline solution (0.9% NaCl) with 1% Tween 80 (50 µL). FUL, animals were instilled with C60 fullerene suspension in 1% Tween 80 (C60, 1 mg/kg BW, total volume: 50 µL). Analyses were performed 24 h after instillation. Arrows represent alveolar edema, circles indicate alveolar collapse, and arrowhead signals septal thickening.
Quantitatively, the number of MNs did not differ between groups, however FUL mice presented a higher number of PMN cells in the lung than CTRL. In this context, 3 days after intratracheal instillation in rats, fullerene C60 in different doses, and times induced the formation of lung inflammatory areas containing neutrophils, eosinophils, and a high number of macrophages together with hemorrhage in alveoli, supporting our findings (Ema et al. 2012). Similarly, an increased lung PMN cell content was observed in mice 24 h after exposure to other pollutants such as residual oil fly ashes, diesel exhaust particles, and fine particulate matter (Laks et al. 2008, Riva et al. 2011, Carvalho et al. 2014).

The percentage of collapsed alveoli in FUL mice was significantly larger than in CTRL, supporting the qualitative microscopic findings and the impairment of lung mechanics. Similar findings were also reported to peak at 24 h after mice exposure to fullerene C60 (Pinheiro et al. 2021). Interestingly, these in vivo results are supported by in vitro simulation modeling of exposure of a two-lipid monolayer to C60 nanoparticles. Monolayer collapse occurs at high surface tensions, possibly enhanced by C60 (Chiu et al. 2012). Putatively, similar mechanisms may compromise pulmonary surfactant in vivo, suggesting that inhaled pollutants may impair properties such as surfactant composition and rheology, which could yield to alveolar collapse, as presently reported (Anseth et al. 2005; Barnoud, Urbini, and Monticelli 2015).

Fullerene C60, as well other pollutants, tend to interact preferentially with cellular and subcellular lipid bilayers and may jeopardize mitochondrial function (Bedrov et al. 2008; Li et al. 2011; Tang et al. 2013; Santos et al. 2014; He et al. 2015; Yang et al. 2016). Probably, the transmembrane electric potential across the inner mitochondrial membrane, generated by protons excess, attracts the negative fullerene nanoparticles (Santos et al. 2014). As the mitochondrial dysfunction is considered a crucial mechanism of nanomaterial toxicity (Santos et al. 2014), the measurement of isolated mitochondria function would contribute substantially to understand their bioenergetics and explore a possible association with lung mechanical function. However, this has not yet been done in ex vivo pulmonary mitochondria after in vivo exposure to C60. Therefore, to obtain isolated mitochondria from lung tissue (Hogeboom, Schneider, and Pallade 1948), we used an upgraded approach of a previously reported method (Maciel et al. 2020).

Exposure to nanoparticles, such as Ag and TiO2, reduce the in vitro activity of all ETC complexes in diverse tissues, including isolated lung mitochondria, and ROS production (Van Der Toorn et al. 2007; Costa et al. 2010; Freyre-Fonseca et al. 2011; Teodoro et al. 2011; Chen et al. 2018). Furthermore, polyhydroxylated fullerene also jeopardizes the mitochondrial respiration chain and damages mitochondrial ultrastructure (Yang et al. 2016). Although the mechanisms triggering these alterations remain unclear, our data suggest that C60 could impair the dynamics and structure of the internal mitochondrial membrane. The present study showed an impaired mitochondrial function 24 h after lung exposure to fullerene, with reduced complex I respiration under pyruvate/malate stimulation (state 2). As the proton leak hypothesis was ruled out, this result could be partially explained by (i) a reduction in electron flow by ETC and a concomitant
Figure 4. Mitochondrial function. (A) State 1 basal mitochondrial respiration; (B) state 2 mitochondrial respiration with pyruvate—malate substrate; (C) state 3 (ADP-stimulated) complex I respiration; (D) state 4 mitochondrial respiration by oligomycin; (E) complex IV respiration and maximum uncoupled respiration (Max Unc) in the presence of TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine)/ascorbate and FCCP (Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone); (F) adenosine triphosphate (ATP) production; (G) extramitochondrial reactive oxygen species (ROS) production; (H) Reason ratio ROS/ATP; (I) oxygen consumption associated with ATP resynthesis; (J) electron leakage; (K) mitochondrial transmembrane potential; (L) mitochondrial swelling. Columns represent mean ± SEM (n = 6 animals per group). CTRL, mice underwent intratracheal instillation of physiological saline (0.9% NaCl) with 1% Tween 80 (50 µL). FUL, animals were instilled with 1% Tween 80 fullerene suspension, 1 mg/kg BW (50 µL). Significantly different groups are indicated by p value.
reduction in the oxygen consumption, and/or (ii) increased permeability to protons (proton leak), impacting on mitochondria function/bioenergetics (Santos et al. 2014). The complex I respiration in the presence of ADP stimulation (state 3) was similar in both groups (Figure 4(C)) and probably results from the spontaneous acceleration of the ETC, as ADP is phosphorylated into ATP and the energy from the intermembrane gradient is lessened.

Another explanation for the increase in oxygen consumption after adding the ATP synthase inhibitor (Figure 4(D)) would be an increase in uncoupled respiration due to proton leakage. This is because contaminants that could increase the oxygen consumption present in the sample, such as Ca$^{2+}$-dependent ATPases and broken mitochondrial F1F0-ATPase, with measurable activities, are unlikely due to the low concentration of mitochondria used (Toth et al. 1990). In addition, the hypothesis of ATP resynthesis by ADP cycling was also discarded in our study (Figure 4(H,I)).

The present study revealed that mice exposed to fullerene C60 produced ATP significantly less than CTRL mice (Figure 4(F)). In this line, oxygraphic traces evidenced decreased oxygen consumption and ATP production after exposure of lung mitochondria to airborne particulate matter (PM$_{10}$) (Delgado-Buenrostro et al. 2013). Reduction in mitochondrial ATP production and increased ROS formation also took place in A549 cells 4 h after exposure to titanium dioxide (TiO$_2$) nanoparticles (Tang et al. 2013), as well as in a murine macrophage cell line originating from Abelson leukemia virus-transformed cell line (RAW 264.7) exposed to nano-TiO$_2$ for 24 h (Chen et al. 2018). Besides, cells gathered by bronchoalveolar lavage disclosed that C60 halts the cell cycle progression in the sub-G1 and G1 phases, which requires a large amount of ATP, thus indicating the failure in ATP production by mitochondria (Park et al. 2010). Therefore, these studies corroborate our findings that fullerene C60 interaction with mitochondria can decrease ATP synthesis.

Our finding of increased production of ROS could be related to the impaired oxygen consumption by ETC experimentally energized with complex I substrates (pyruvate–malate). About 5% of the electrons that flow along the electron transport chain leak into the mitochondrial matrix and form a superoxide anion that is quickly converted into hydrogen peroxide by the enzyme superoxide dismutase and permeates the membrane (Quinlan et al. 2012; Moreno-Sánchez et al. 2013). Interestingly, the pathophysiological relevant sites of ROS generation in the mitochondrial ETC are: (i) the flavin mononucleotide (FMN) group of complex I (where electrons leakage occurs during the reverse electron flow), and (ii) the complex II (Liu, Fiskum, and Schubert 2002; Moreno-Sánchez et al. 2013). However, our study demonstrated that electron leakage did not occur under our experimental conditions. Moreover, our results of pulmonary inflammation could be associated with fullerene C60-induced oxidative stress by mechanisms involving mitochondrial dysfunction, such as NLRP3 inflammasome activation positively regulated by reactive oxygen species (ROS) (Zhou et al. 2011).

In contrast with other studies (Santos et al. 2014; Yang et al. 2016), we did not find fluctuations of transmembrane potential or mitochondrial swelling (Figure 4(K,L)). Thus, possibly the exposure time used in this study was not long enough to cause changes in the mitochondrial structure or morphology.

5. Conclusions
Exposure to fullerene C60 jeopardized lung function, leading to impairment of lung parenchyma mechanical parameters, microscopically damaged pulmonary tissue, and triggered lung inflammatory cell influx at 24 h after installation. As a novelty, fullerene C60 also compromised lung mitochondrial function by reducing O$_2$ consumption by electron transfer chain and ATP production, as well as by increasing production of extramitochondrial ROS.

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