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Unique Heterogeneous Topological Pattern of the Metabolic Landscape in Rabbit Fetal Lungs Following Tracheal Occlusion

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Abstract

Introduction—Fetal tracheal occlusion (TO) is currently an experimental approach to drive accelerated lung growth. It is stimulated by mechanotransduction that results in increased cellular proliferation and growth. However, it is currently unknown how TO affects the metabolic landscape of fetal lungs.

Materials and Methods—TO or sham was performed on fetal rabbits at 26 days followed by lung harvest on day 30. Mass spectrometry was performed to evaluate global metabolic changes. Fluorescence lifetime intensity microscopy (FLIM) was performed to estimate local free/bound NADH relative ratio as an indicator of aerobic glycolysis versus oxidative phosphorylation (glycolysis/OXPHOS).

Results—TO results in a metabolic shift from tricarboxylic acid cycle towards glycolysis. FLIM reveals uniform structures in control lungs characterized by similar ratios of free/bound NADH indicating a homogenous topological pattern. Similar uniform structures are observed in shams with some variability in glycolysis/OXPHOS ratio. In contrast, lungs following TO demonstrate different types of unique distinct topological zones: one with enlarged alveoli and a shift towards glycolysis; the other maintains balance between glycolysis/OXPHOS similar to control lungs.

Conclusion—We demonstrate for the first time a unique variable topological pattern of metabolism in fetal lungs following TO with a wide variation of metabolism between zones.

Keywords

Heterogeneous metabolic zones; Tracheal occlusion; accelerated lung growth; congenital diaphragmatic hernia; FLIM

Introduction

Congenital diaphragmatic hernia (CDH) is a multi-genetic pathological condition whereby branching morphogenesis and embryonic pulmonary vascular development are concurrently affected with an arrest during the pseudoglandular stage of development [1]. Tracheal occlusion (TO) is an experimental approach offered to the most severe cases of CDH to drive accelerated fetal lung growth [2]. Deprest et al., demonstrated that percutaneous fetoscopic endoluminal tracheal occlusion (FETO) can be performed successfully and is associated with an apparent increase in neonatal survival [3]. However, clinical response and outcomes following TO have been variable.

Fetal pulmonary growth is a classic example of mechanotransduction-dependent cellular proliferation. Growth occurs due to a normal transpulmonary pressure gradient generated by lung fluid production against the fixed resistance of the glottis and is accentuated by fetal breathing movements [4]. The resulting transpulmonary pressure gradient actively distends the lung and passively distends the chest wall to a volume equivalent to the functional residual capacity (FRC) [5]. The aforementioned developmental role of airway distension forms the basis of pulmonary hypoplasia seen in some clinical scenarios. For instance, in oligohydramnios, decreased amniotic fluid is the reason for the lack of airway distention, however, in other scenarios like congenital diaphragmatic hernia, skeletal dysplasia and neuromuscular paralysis of the diaphragm, lack of airway distention is secondary to alterations in the mechanical environment of the thorax [6,7].

Konstantinos et al investigated whether lung growth after TO is a mere function of increased pressure within the occluded lungs. They demonstrated that substituting tracheal fluid in occluded sheep by an equal volume of saline did not result in the same amount of lung growth despite a constant intratracheal pressure between 3 and 5 torr, thus raising the question of a biological role of the natural lung fluid [8]. Moreover, they reported that following tracheal ligation, an abrupt increase occurs in the net lung fluid production of up to threefold in volume. Such increase occurs after 3 days possibly secondary to increased secretory cell mass attributed to cell proliferation. Other investigators similarly reported that two-thirds of the increase in pulmonary DNA content following tracheal ligation occurs between days 2 and 7 [9]. Such phenomena may be due to the time needed to allow for accumulation of enough intratracheal fluid to generate the mechanical forces necessary to sufficiently stimulate mechanotransduction.

Mechanical stretch induced by TO has been shown by quantitative PCR to result in an increased expression of some of the genes involved in alveorization. For example, genes encoding matrix components like elastin (*ELN*) and Tenascin C (*TNC*), those regulating the assembly and stability of the extra cellular matrix like lysyl oxidase (*LOX*) and fibulin 5 (*FBLN5*), controlling rearrangements of actin-cytoskeleton like integrin subunit alpha 6 (*ITGA6*), integrin subunit beta 1 (*ITGB1*), and drebrin 1 (*DBN1*), and those encoding matrix metalloproteinases (*MMP2*) and tissue inhibitors of metalloproteinases (*TIMP2*) are overexpressed following TO in relation to the homeobox genes [10].

Pulmonary development and growth is a dynamic process that requires the support of a concomitantly dynamic metabolism to match the ever-increasing needs of growth including proliferation, differentiation, and biosynthetic demands. Therefore, cells preparing for proliferation are required to utilize a unique repertoire of metabolic processes with a change to a “Warburg-like metabolism” which is dependent on aerobic glycolysis to achieve growth [11]. The major function of aerobic glycolysis in proliferating cells is to maintain a high level of glycolytic intermediates to support anabolic reactions in these cells [12].

In contrast to conventional glycolysis, actively growing cells will shift the glycolytic flux towards lactate production thus supporting the pentose phosphate shunt by generating large amounts of the NADPH. Therefore, in proliferating tissues there is a very specific metabolic landscape including: high level aerobic glycolysis, along with lactate production and cellular excretion. This metabolic shift allows for both adequate ATP production and a surplus of carbon and NADH for growth [13].

It is currently unknown how TO affects the metabolic landscape of fetal lungs and whether TO will lead to a similar adoption of glycolysis to support the actively growing cells. The aim of this study is to examine the global and local metabolic changes in normal rabbit fetal lungs following TO.

Methods

Fetal Rabbit Tracheal Occlusion Model

Time-dated pregnant New-Zealand white rabbits were obtained at 17 to 19 days gestational age from Charles River Laboratory and housed in separate cages under standard laboratory conditions with free access to water and chow. Rabbits were allowed to acclimate for 7 days to compensate for the high altitude in Denver and conform to our institutional animal care and use committee (IACUC) regulations. On the day of surgery, each doe was pre-medicated with ketamine/xylazine (15 mg/kg and 2.5mg/kg respectively) intramuscularly, medroxyprogesterone acetate, 7 mg (Depot-Provera) and penicillin G, 300,000 units IM. Tracheal Occlusion was performed as previously described [14, 15]. Briefly, pregnant rabbits at 26 days of gestation were maintained on general anesthesia using isoflurane (2% to 3%) in oxygen at 1 L/min, using a facemask. Continuous maternal heart rate and oxygen saturation were monitored with a pulse oximeter. All operations were carried out under aseptic conditions. The pregnant uterus was exposed through a 5cm lower midline laparotomy. Uterine interventions were performed using micro-instruments and an operating microscope. Only two fetuses per doe were operated on; corresponding ovarian end position on opposite horns (TO and sham). When the ovarian end fetus was smaller, a larger fetus towards the ovarian end and its corresponding position fetus on the opposite horn were selected for surgery. Fetal position was determined by gentle palpation followed by a 1–1.5cm transverse hysterotomy on the anti-mesometrial border of the uterus. Hemostasis was achieved using a needle tip cautery followed by six 4–0 silk stay sutures placed on the border of the uterine incision to prevent membrane dislodgment. Fetal head and neck were gently delivered through the hysterotomy. Tracheal occlusion was performed via a midline neck incision. The trachea was dissected and separated from the esophagus and a single 4–0 silk stitch was passed around the trachea and gently tied. A sham operation was performed

on a fetus in corresponding position on the opposite horn with exposure and dissection of the trachea, but no ligation. Following surgery, fetuses were gently manipulated back into the uterine cavity. Three milliliters of warmed lactated Ringers solution were infused into the amniotic cavity to maintain amniotic fluid volume followed by closure of the hysterotomy using a running 4–0 silk suture. The uterus was placed back into the abdominal cavity and the abdomen closed in layers. All animals received Bupivacaine 0.5% (1–2mg/kg) and Buprenorphine SR LAB (0.15mg/kg) IM along the incision for post-operative analgesia in addition to 10ml/kg of normal saline subcutaneously.

Harvest: All fetuses were harvested by Cesarean section on day 30. Does were submitted to the same premedication, anesthesia and positioning. Three fetuses per doe (tracheal occlusion, sham, and un-operated pregnancy control) were delivered via a cesarean section. Immediate euthanasia was performed using approved methods. Successful TO was confirmed by naked eye examination documenting larger lungs filling the chest cavity, with no apparent evidence of tracheal injury, prior to tissue harvest. Lung tissue was immediately harvested, flash frozen in liquid nitrogen and then stored in -80°C (for metabolomics and FLIM) or fixed in 4% paraformaldehyde for histological and morphometric analysis.

Magnetic Resonance Imaging evaluation: Contrast-enhanced magnetic resonance imaging (MRI) was performed at day 30 after surgery on a separate cohort of 10 animals (4 TO, 4 shams, and 2 controls). The pregnant rabbit was anesthetized using a ketamine/xylazine injection. An intravenous bolus injection of gadolinium contrast (*0.4 mmol/kg MultiHance, gadobenate dimeglumine, Bracco Diagnostics*) was administered to the pregnant rabbit followed by a 10min lag for circulation and placental penetration. Fetuses were euthanized using an intraperitoneal injection of fatal plus (0.5ml) while still inside the uterus. Following euthanasia, body weight was obtained followed by MRI scans. The pup was oriented onto a cardboard strip for stabilization and placed into a Bruker 4.7 Tesla MRI scanner equipped with a 36-mm diameter volumetric RF coil. High-resolution rapid acquisition with relaxation enhancement proton density weighted MR images were obtained for the volumetric assessment of fetal lungs: field of view 4 cm, slice thickness 0.7 mm, TR/TE= 3000/30 msec, flip angle 180 degrees, matrix size 256×256, total acquisition time 6 min. All images were acquired in axial and coronal plane, with in-plane resolution of 75 microns. All acquisition and image analysis were performed using Bruker ParaVision v4.1 software. Total lung volumes were quantified/visualized by placing a hand-drawn region of interest (ROI) over the lung and mediastinal structures on each anatomical slice. Total lung volumes-to-body weight ratios were subsequently calculated.

Mass Spectrometry-based metabolomics Analysis

All solvents were Optima grade (Fisher Scientific). Frozen (wet) lung samples were weighed to the nearest 0.01 mg. Extraction concentrations were normalized using the wet weights to a concentration of 10 mg of frozen tissue per mL of extraction buffer. Frozen samples were subjected to metabolite extraction with ice-cold extraction buffer (methanol:acetonitrile:water 5:3:2) in the presence of 1mm glass beads by agitation at 4°C for 30 min followed by centrifugation at 10,000 *g* for 10min at 4°C . Ten μl of supernatants were injected into a Thermo Vanquish UHPLC coupled to a Thermo Q Exactive mass

spectrometer and run on a Phenomenex Kinetex C18 column (150 × 2.1 mm i.d., 1.7 μm) at 250 μl/min (isocratic gradient: 5% acetonitrile, 95% H₂O). Samples were run in positive and negative ion modes, 3min each, as described [16,17]. Phases for positive mode were supplemented with 0.1% formic acid. Phases for negative mode were supplemented with 5 mM NH₄OAc. The mass spectrometer was operated in Full MS mode at 70,000 resolution in the 60–900 m/z range using electrospray ionization. Raw files were converted to mzXML format using MassMatrix (Case Western Reserve University) and metabolite assignments were subsequently determined using Maven (Princeton University) and assignments confirmed against a metabolite library (IROATech, Sigma Aldrich). Data obtained were plotted without correction since normalization by weight was performed at the stage of metabolite extraction.

Fluorescent Lifetime Intensity Microscopy (FLIM)

FLIM was performed to detect changes in metabolism in fifteen different areas of each lung lobe characterized by levels of free vs. bound NADH in fresh lung pieces using a Zeiss 780 laser-scanning confocal/multiphoton-excitation fluorescence microscope with a 34-Channel GaAsP QUASAR Detection Unit and non-descanned detectors for two photon fluorescence (Zeiss, Thornwood, NY) equipped with a ISS A320 FastFLIM box and a titanium:sapphire Chameleon Ultra II (Coherent, Santa Clara, CA). A dichroic filter (496 nm, Semrock Inc, Rochester, NY) was used to separate the fluorescence signal from the laser light and the fluorescence. For the acquisition of FLIM images fluorescence was detected by a photon-counting PMT detector (H7422p-40; Hamamatsu). Images of the lung were obtained with Vista Vision software by ISS in the 256×256 format with a pixel dwell time of 6.3 μs/pixel and averaging over 30 frames. FLIM calibration of the system was performed by measuring the known lifetime of the fluorophore fluorescein with a single exponential of 4.0 ns [18]. The phasor transformation and data analysis were carried out using Global SimFCS software (Laboratory for Fluorescence Dynamics, University of California, Irvine) as described previously [19]. Fractional analysis of free vs. bound NADH in each pixel was determined based on fluorescence lifetime corresponding to 0.4 ns (free) and 3.4 ns (bound) for NADH.

Statistical Analysis

Data were expressed as means ± SD. Statistical differences between groups were determined by one-way ANOVA Tukey correction for multiple comparisons using Prism 6.0 Software (GraphPad, San Diego, CA). Statistical differences were determined as significant for P < 0.05. One-way ANOVA for metabolomics results was performed using MetaboAnalyst (without post hoc normalization of results) and heat maps prepared using GENE-E (The Broad Institute).

Results

Fetal Surgery Outcomes

Twelve pregnant does were operated on with a total number of 19 fetuses. One pregnant doe was euthanized on post-operative day 1 due to persistent vaginal bleeding and 1 doe had severe diarrhea. 14 fetuses (7 shams and 7 TO) survived till harvest (74% survival).

Tracheal occlusion induced lung growth documented using traditional methods

Similar to previously reported fetal rabbit tracheal occlusion models, our occluded fetuses demonstrate accelerated lung growth as evidenced by an increase in the wet lung-to-body weight ratio (LBWR) [6, 11]. TO fetuses (n=5) demonstrate a LBWR of 0.05 compared to 0.025 and 0.026 for sham (n=6) and control (n=3) fetuses, respectively ($p=0.0003$ and $p=0.0009$ one-way ANOVA) (Figure 1). Moreover, morphometric analysis demonstrates an increase in the radial alveolar counts in the TO group compared to the sham and control groups (n=3) (7.9 Vs. 6 and 5.2, $p=0.018$ and $p=0.0001$, respectively, one-way ANOVA) (Figure 2) [20].

Magnetic Resonance Imaging 3-Dimensional Lung Volumes.

TO results in a significant 3.5-fold increase in the total lung volume $3343 \pm 508 \text{ mm}^3$ (n=4) versus $974 \pm 81 \text{ mm}^3$ in sham (n=4) and $1300 \pm 157 \text{ mm}^3$ in controls (n=2) ($p<0.0001$ and $p<0.003$, respectively, un-paired t-test). In order to correct for fetal body weight, total lung volume-to-lung body weight ratio was calculated. TO results in a significant increase in the total lung volume – body weight ratio compared to shams and controls (85.59 vs. 32.63 and 39.185, $p<0.0001$ and $p=0.0002$, respectively, one-way ANOVA) (Figure 3).

The Global Metabolic Landscape Following Tracheal Occlusion

Untargeted high-throughput metabolomics performed using ultra high-pressure liquid chromatography coupled online to mass spectrometry (UHPLC-MS) demonstrates a shift from mitochondrial metabolism to glycolysis following TO in comparison to sham and controls as judged by significant decreases in fumarate and citrate, which are considered as some of the key metabolites of the tricarboxylic acid cycle (TCA) (Figure 4). Moreover, since TO results in accelerated lung growth, it is not surprising to observe an overall significant reduction in many metabolites, particularly free amino acids used as building blocks in protein synthesis, following TO (Figure 5).

Unique Heterogeneous Metabolic Topological Zones Formed by Tracheal Occlusion – Fluorescent Lifetime Intensity Microscopy (FLIM)

Analysis of fifteen different random images in the respective fetal lung lobes reveals similar structure in both control and sham lungs with a homogeneous metabolic landscape. Sham lungs demonstrate a non-statistically significant increase in the free/bound NADH ratio compared to controls (0.53 ± 0.05 compared to 0.35 ± 0.07 , respectively). When evaluating all TO zones, there is further increase in the free/bound NADH ratio compared to shams (0.92 ± 0.11 compared to 0.53 ± 0.05 , respectively, $p=0.038$, one-way ANOVA). However, TO demonstrates at least two unique distinct types of zones; one zone type structurally looks similar to both controls and shams and has a similar free/bound NADH ratio to controls (0.35 ± 0.04 compared to 0.35 ± 0.07 , respectively). The second type of zone demonstrates evidence of alveolar structural enlargement and is characterized by a significant increase in the free/bound NADH ratio indicative of a shift from OXPHOS towards glycolysis (1.45 ± 0.19 compared to 0.35 ± 0.07 in control, $p<0.0001$, one-way ANOVA) (Figure 6).

Furthermore, we noticed an intra-alveolar longer lifetime signal that is most likely coming from the surfactant layer (cyan and magenta cursors on the phasor plots). This lifetime

signal was comparable in the sham and control groups ($9.16 \pm 0.98\%$ of covered pixels vs. $6.48 \pm 0.80\%$, respectively). However, it decreased significantly in the TO group ($3.39 \pm 0.53\%$ of covered pixels compared to $9.16 \pm 0.98\%$ and $6.48 \pm 0.80\%$, for controls and shams, $p=0.027$ and $p<0.0001$, respectively, one-way ANOVA). After image separation, according to the size of alveoli, the first type of zones without evidence of alveolar structural enlargement and a balance between OXPHOS and glycolysis demonstrate comparable “surfactant lifetime signal” to controls ($6.26 \pm 0.93\%$ of covered pixels compared to $6.48 \pm 0.80\%$, respectively). However, the second type of zones with structural alveolar enlargement and shift in the metabolic landscape to glycolysis, demonstrate a significant decrease in the “surfactant lifetime signal” compared to controls ($0.74 \pm 0.15\%$ of covered pixels vs. $6.48 \pm 0.80\%$, respectively, $p<0.0001$, one-way ANOVA).

Discussion

In this manuscript, we present for the first time a novel concept of formation of unique heterogeneous topological zones with variable patterns of metabolism in normal rabbit fetal lungs following TO. On a global level, TO results in a significant decrease in almost all metabolites compared to shams and controls, which is not surprising given the demands for a metabolic burden to achieve lung growth. Moreover, common metabolic changes in fetal lungs after TO are represented by an increase in glycolytic metabolism and a decrease in the activity of the TCA cycle as evidenced by a shift in the fate of pyruvate towards lactate instead of citrate and forward to fumarate, which constitute some of the key metabolites of the TCA cycle.

More importantly, FLIM analysis of the local metabolic changes in fetal lungs after TO reveals formation of at least two distinct types of metabolic zones. The first type of zone is characterized by a significant increase in the free/bound NADH ratio reflecting an increase in glycolysis instead of oxidative phosphorylation. The second type of metabolic zone is characterized by similar free/bound NADH ratio as seen in control and sham lungs without a significant increase in glycolysis.

Our observed phenomenon of heterogeneous topological zones in the fetal lungs after TO raise a number of fascinating questions. First, when do such topological zones appear and how long will they persist? Second, are these heterogeneous topological patterns isolated to metabolism, or are they also observed in cellular/tissue stress, extent of lung tissue damage, the associated subsequent inflammation, angiogenesis/vasculogenesis and structural lung changes among others? Third, what are the mechanisms involved in the formation of this variable topological landscape? And finally, what is the biological/clinical relevance of the formation of these unique topological zones after TO?

Lung fluid accumulation in the occluded fetal airway may play a dual role in the process of lung growth. Firstly, it will result in mechanical stimulation of lung growth [21] and secondly, it may induce some form of local cell/tissue stress and damage. We suggest that the specific action of mechanical forces in different zones of the lung could play a significant role in the formation of a heterogeneous metabolic landscape in the fetal lungs following TO.

It is well established that one of the functions of mast cells is to sense the surrounding environment [22,23]. However, could the lung mast cells serve as a pressure and 3-Dimensional (3-D) geometry sensing mechanism after TO? We suggest that these cells may potentially gauge the excessive mechanical distension of the lung and respond to such stimulation. Moreover, mechanical stress may represent a priming signal for mast cell activation. An alternative pathway for involvement of lung mast cells in the modulation of lung tissue response after TO could be mast cell activation via Damage Associated Molecular Pattern Substances (DAMPs).

Furthermore, lung fluid accumulation after TO will result in a variable change in the 3-D geometry of fetal lungs in different zones dependent on the local tissue compliance and the degree of fluid accumulation. This may explain the variable structure in the different TO zones seen in our experiments. Therefore, geometry sensing mechanisms may be involved in the formation of a variable metabolic landscape following TO.

A unique metabolic milieu in some fetal lung zones will form a different cellular and extracellular metabolic environment that will create a specific “niche” for the resident cells in these zones. The influence of these metabolic “niches” on cells, signals and systems might lead to the formation of a common vector of biological response unique to each individual zone. The specific character of metabolic “niche” in different fetal lung zones could delineate distinctive features in the hierarchy of communicating mechanisms involved in signaling, cell sorting, and differentiation and compartmentalization during accelerated fetal lung growth induced by TO. For instance, a specific metabolic landscape, for example, has a very strong influence on the endothelial cell phenotype during angiogenesis [24]. Aerobic glycolysis in endothelial cells promotes a “tip cell phenotype”, thus facilitating endothelial cell migration, vasculogenesis and angiogenesis.

In conclusion, in this paper we demonstrate for the first time a unique phenomenon of a variable metabolic landscape in normal fetal rabbit lungs following TO. We suggest that following TO, fetal lungs will look like a heterogeneous mosaic with a variable degree of 3-D change in geometry, dissimilar response to the increased hydrostatic pressure, variable changes in metabolism and different damage/cellular stress, among others that leads to the formation of a variable vector of biological response in different zones. A limitation to this study is that these results were observed following TO in normal fetal rabbits without a diaphragmatic hernia defect. However, is it possible that similar results with a unique metabolic fingerprint will be observed in CDH fetal rabbits subjected to tracheal occlusion? And could the formation of such unique heterogeneous metabolic zones in the fetal lungs be one of the reasons responsible for the inconsistent pulmonary vascular response and outcomes in severe congenital diaphragmatic hernia treated by TO? Future experiments in our laboratory will focus on characterizing the temporal metabolic response following TO in normal fetal rabbits and examine the metabolic landscape in congenital diaphragmatic hernia rabbit fetuses treated with TO.

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Fig. 1. Tracheal occlusion (TO) induced lung growth. Tracheal occlusion results in a statistically significant increase in the lung-to-body weight ratio (0.05) compared to shams (0.025) and controls (0.026) ($p = 0.0003$ and $p = 0.0009$, respectively, one-way ANOVA).

Fig. 2. Morphometric analysis after tracheal occlusion (TO) – radial alveolar counts (RAC). RAC was calculated as previously de-scribed by Emery and Mithal [21]. Tracheal occlusion results in a statistically significant increase in RAC compared to shams and controls (7.9 vs. 6 and 5.2, $p = 0.018$ and $p = 0.0001$, respectively, one-way ANOVA).

Fig. 3. Magnetic resonance imaging (MRI). Total lung volume-to-body weight ratio. Representative proton density-weighted MRI on tracheal occlusion (TO), sham and control fetuses. The multi-slice region of interest (ROI) analysis was used to quantitatively assess total lung volume-to-body weight ratio of each animal. TO results in a significant increase in the total lung volume-to-body weight ratio compared to shams and controls (85.59 vs. 32.63 and 39.185, $p < 0.0001$ and $p = 0.0002$, respectively, one-way ANOVA).

Fig. 4.

Profiling of glucose metabolism by high-throughput metabolomics (ultra-high-pressure liquid chroma-tography coupled online to mass spectrometry). Tracheal occlusion (TO) results in a shift from mitochondrial metabolism to glycolysis compared to controls and shams as evidenced by significant decreases in tricarboxylic acid cycle (TCA) metabolites fumarate and citrate.

Fig. 5.

Heat map with hierarchical clustering of metabolites significantly altered following tracheal occlusion (TO). TO results in an overall significant reduction in many metabolites, particularly free amino acids used as building blocks in protein synthesis. Metabolites shown here have $p < 0.05$ as determined by one-way ANOVA.

Fig. 6. Fluorescent lifetime intensity microscopy (FLIM) after tra-cheal occlusion (TO). Representative FLIM images of control, sham and TO lungs. Control and sham lungs demonstrate similar histological structures with a homogeneous metabolic landscape. TO animals demonstrate two distinct types of zones. Phasor plot analysis and graphical representation of free/bound NADH of controls, shams and combined regions of TO animals demonstrate a nonstatistically significant increase in the free/bound NADH ra-tio in shams compared to controls (0.53 ± 0.05 compared to 0.35 ± 0.07 , respectively). When evaluating all TO zones, there is fur-ther increase in the free/bound NADH ratio compared to shams (0.92 ± 0.11 compared to 0.53 ± 0.05 , respectively, $p = 0.038$, one-way ANOVA). However,

TO demonstrates at least two unique distinct types of zones; one zone type structurally looks similar to both controls and shams and has a similar free/bound NADH ratio to controls (0.35 ± 0.04 compared to 0.35 ± 0.07 , respectively). The second type of zone demonstrates evidence of alveolar structural enlargement and is characterized by a significant increase in the free/bound NADH ratio indicative of a shift from OXPHOS towards glycolysis (1.45 ± 0.19 compared to 0.35 ± 0.07 in control, $p < 0.0001$, one-way ANOVA).

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