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Emphysema is – at the most – only a mild phenotype in the Sugen-hypoxia rat model of pulmonary arterial hypertension

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To the editor:

Translational research is essential to develop strategies for the treatment of pulmonary arterial hypertension (PAH) using animal models which reproduce the severity, the progressive nature and resistance to treatment of human PAH, including severe arterial remodeling and progressive right ventricular (RV) failure (1).

We read with interest the letter by Kojonazariov *et al.* who propose to have found "severe emphysema in the SU5416/Hypoxia (SuHx) rat model of pulmonary hypertension"(2). The authors report that Wistar-Kyoto rats exposed to the combination of VEGFR2 inhibition by SU5416 and chronic hypoxia had moderately increased RVSP and RV mass compared to normoxic untreated animals(2). They applied in vivo micro-computed tomography (CT) to demonstrate an increase in lung volume and decreased lung density, an unaltered amount of lung tissue, but an increased air-to-tissue ratio, and claim these findings were confirmed by histological analysis, including mean linear intercept as surrogate of emphysema(2). Indeed, SU5416 has been previously shown to induce emphysema in normoxia(3), but this required repetitive SU5416 dosing (3 times weekly over 3 weeks) and occurred more predominantly in rats younger than 4 weeks of age (Norbert Voelkel, personal communication). In addition, emphysema could be negated, at the cost of the development of severe angioproliferative hypertension, by concomitant exposure to hypoxia(4).

The SuHx model, combining a one-time subcutaneous injection of Sugen (SU5416), that blocks VEGF receptor 2 but also other tyrosine kinases, followed by 2-4 weeks of hypoxia(5, 6)(7), is the most accepted rodent model for pulmonary arterial hypertension (PAH). Although several modifications of the SuHx model exist, the most commonly used study design is the one-time injection of 20mg/kg SU5416 s.c. in 6-8 week old Sprague Dawley rats /body weight (180-200g), followed by 3 weeks hypoxia (10% oxygen), and subsequently a 1-10 week period in room air, until heart-lung function and morphology is assessed.

Experiments in male Sprague Dawley rats demonstrated that RV systolic pressure (RVSP) increases over 90 mmHg in response to SuHx at three(5, 6) or four weeks hypoxia(7), then decreases somewhat in some studies but remains elevated at 73 mmHg(7), 91 mmHg(6) and 96 mmHg upon return to normoxia for additional four(7), six or ten weeks(5, 6), respectively (**Table**). Importantly, at six(6) and ten weeks(5) after the end of hypoxia, plexiform lesions that are very

similar to such lesions in humans (IPAH), were frequently found in SuHx rats(5, 6), in addition to concentric medial hypertrophy of small- and medium-sized pulmonary arteries(6). Legchenko et al. demonstrated that RV failure develops between 1 and 6 weeks after the end of 3 weeks hypoxia in SuHx rats, together with progression of pulmonary vascular disease (PVD), loss of peripheral pulmonary arterioles, and a metabolic switch in the RV(6).

The advantages of the SuHx rat versus most other rat PAH models such as monocrotaline (MCT) were highlighted by a group of experts and include the intensification of vascular remodeling process, leading to the appearance of human-like plexiform lesions, and the virtual unresponsiveness to current PAH treatments, correlating well with the common unresponsiveness of PAH to therapy in humans(1).

Importantly, “severe emphysema” has not been previously observed in the SuHx rat model of PAH. Based on the report by Kojonazariov et al.(2), we have conducted not only a literature search but also analyzed lung histology from different SuHx rat studies in established laboratories (**Table, Figure**). In contrast to Kojonazariov et al., we did not find any severe or even moderate emphysema in any of the SuHx models analyzed. **(Figure 1; Table**). Mean linear intercept (MLI) as a surrogate for alveolar enlargement was not significantly different in SuHx vs. untreated control lungs (ConNx) from Sprague Dawley rats obtained from Charles River, Sulzfeld, Gemany (**Figure 1A, 1B**). The Stewart group measured MLI in Sprague Dawley rats obtained from Harlan, Indianapolis, USA, and found a mild (18%) increase in MLI in SuHx vs. naïve no-vehicle control (ConNx) rats (8). (**Table, Figure 1C, 1D**).

Several reasons may explain the differences in the above findings and the recent report by Kojonazariov et al.(2): This group studied Wistar Kyoto (2) rather than Sprague Dawley rats that have been used by most other groups, and observed a much weaker PAH hemodynamic phenotype (RVSP 55mmHg, at 3+2 weeks SuHx) when compared with many other publications (i.e., RVSP 91-107mmHg upon return to normoxia for 4-10 weeks; **Table)**(5-8). It is possible that the Wistar rat strain may be more prone to emphysema after SuHx, however, Dean *et al.* did not report any emphysema-like lung phenotype in female Wistar rats exposed to SuHx (RVSP 55 mmHg)(9). The Stewart group compared the response to SuHx in several different strains of rats: While Fischer and Sprague Dawley rats developed similar increases in RVSP to ~100 mmHg(8),

Lewis rats exhibited no significant increase in RV pressure, highlighting the importance of background in determining the phenotype in the SuHx model.

In addition to the above strain differences, technical factors may also contribute to the differences observed between research groups. While we congratulate Kojonazariov et al.(2) on performing *in vivo* micro-CT to quantify the air-to-lung-tissue volume ratio as a surrogate of air space disease (emphysema), it remains unclear whether, and - if so - how the images were gated to the respiratory cycle. From the clinical experience, expiratory chest CT lung images must be interpreted with caution, and of course yield lower such ratio numbers than end-inspiratory CT images. As well, valid measurement of MLI in *ex vivo* histological studies critically depends on standardized tracheal inflation pressure and volume, in the absence of a pleural leackage.

In summary, based on a literature search and the analysis of our own SuHx rat studies (**Table 1; Figure 1**), we cannot confirm the presence of moderate or severe emphysema in the established Sugen-hypoxia rat model of PAH. At the most, there may be mild enlargement of intraalveolar spaces depending on rat strain, number of SU5146 doses and timing of lung harvest. In contrast, there is ample evidence that repetitive SU5416 injections alone (i.e. blockade of VEGFR2 and other kinases) in the absence of hypoxia, can produce an emphysema-like lung phenotype, but the latter mainly occurs in younger rats where postnatal lung development may still be ongoing(3). Our data provide evidence that the Sugen-hypoxia (SuHx) rat model, when yielding RVSP consistently > 60 mmHg, using adequate controls and standardized lung inflation, is currently one of the best rodent models to study PAH and PVD, including mechanisms of vessel loss and RV failure, and as such lacks a biologically relevant emphysema-like lung phenotype.

Conflicts of interest

D.J.S. is a founding scientist, consultant for and has equity interest in Northern Therapeutics Inc. G.H. holds a patent application (USPTO no. 1289344) and an investigational new drug application (IND no. 105,428) related to the use of PPARγ agonistic agents for the treatment of pulmonary hypertension.

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FIGURE LEGENDS

Figure 1. Emphysema is – at the most – only a mild phenotype in the SuHx rat model of pulmonary arterial hypertension (PAH).

(**A**) Representative pictures of Elastica van Gieson staining of the lungs from control normoxia (ConNx) and Sugen hypoxia (SuHx) rats. Scale bar: 200 µm. (**B**) Quantification of mean linear intercept (MLI) shows that there is no significant difference between ConNx group and SuHx group. (**C**) Representative pictures of H&E staining of the lungs from ConNx and SuHx rats from the Stewart group. Scale bar: 200 µm. (**D**) Quantification of MLI shows that MLI has a significant but mild increase in SuHx compared to ConNx rats. The MLI (also called air space chord length, Lm), as a surrogate of air space diameter, was determined as follows:

A/B: The left lung was tracheally filled by 1:1 mix of saline and cryofixative (Tissue-Tek OCT), and snap frozen in liquid nitrogen. Lung cryosections (5 µm) were stained with Elastica van Giesson for morphometric analysis. Six to eight random fields (100x magnification) for each rat were analyzed for mean linear intercept. Five to ten lines of 800 um length were drawn per image, alveolar intercepts per line were counted and mean linear intercept was calculated.

C/D: The left lobe of the lung was inflated via the trachea with 1:1 OCT/saline solution (Tissue-Tek OCT) and then removed. The left lobe was then cut into thick cross sections and fixed in 4% paraformaldehyde (PFA) for 24 h, rinsed and washed in PBS for 8 hr and stored in 70% ethanol until the day of paraffin embedding. Eight random high power fields (100 x magnification) for each rat were analyzed for mean linear intercept. Three lines of 1000um length were drawn per image, alveolar intercepts per line were counted and mean linear intercept was calculated.

SuHx group: rats injected once with SU5416 20-25 mg/kg/dose s.c., and subsequently exposed to chronic hypoxia, i.e. 10% oxygen. **A/B**: 4 weeks hypoxia, followed by 6 weeks in room air and lung harvest. **C/D**: 3 weeks hypoxia followed by lung harvest). Rat supplier for **A/B**: Charles River, Sulzenfeld, Germany. Rat supplier for **C/D**: Harlan, Indianapolis, Indiana, USA. For additional experimental details, see Table. Columns and error bars represent mean±SEM, n=5-6 per group. Non-parametric Mann-Whitney test. ** $p < 0.01$; n.s., not significant. Scale bar = 200µm

TABLE with legend

Table, see subsequent page

Reference	Rat Strain	Gender, age, weight at Su5416 injection	Su5416 brand, single dose s.c. (solvent)	Duration of hypoxia + normoxia (weeks)	RVSP (mmHg)	Emphysema reported	Re-analysis/Comment
Kojonazarov et al. AJRCCM, 2019 (2)	Wistar Kyoto	Not reported	Brand not reported, 20mg/kg (solvent ?)	**3+2**	~55 ± 6	Yes, "severe"	SuHx vs. ConNx comparison: MLI (histology): ~45 vs 35μm, Air-to-lung tissue volume ratio (CT): ~2.4 vs. 1.6; $p < 0.001$
Dean et al. AJRCMB, 2018 (9)	Wistar Kyoto	Female (no more information)	Brand not reported, 20mg/kg (solvent ?)	**2+6**	~50	No	
Legchenko et al. Sci Transl Med, 2018 (6)	Sprague Dawley[1]	Male, 6-8wks old, 180-200g	Sigma, 20mg/kg (DMSO)	3+1, 3+3, **3+6**	91 ± 7	No	Plexiform lesions at 3+6 weeks very similar to human plexiform lesions. RV failure occurs between 3+1 and 3+6 weeks (MRI, closed-chest right and left heart catheterization).
Dabral et al. Eur Respir J, 2016 (10)	Not reported	Not reported, 200-250g	Brand not reported, 20mg/kg (DMSO)	**5+0**	~52 ± 2	No	
Bogaard group, unpublished data	Sprague Dawley[1]	Male, <200g	Tocris, 25mg/kg (CMC)	**4+6**	72 ± 2	No	SuHx vs. ConNx comparison: MLI (histology) 47 vs. 46μm, n.s.; see Figure 1A, 1B
Stewart group, unpublished data	Sprague Dawley[2]	Male, 6-7wks old, 175-200g	Tocris, 20mg/kg (CMC)	**3+4**	105 ± 10	Yes, "mild"	SuHx vs. ConNx comparison: MLI (histology) 55 vs. 46μm, $p < 0.01$; see Figure 1C, 1D
Jiang et al. AJRCMB, 2016 (8)	Sprague Dawley[2]	Male, 6-7wks old, 175-200g	Tocris, 20mg/kg (CMC)	**3+5**	104 ± 13	No	Assessed vascular remodeling.
De Raaf et al. Eur Respir J., 2014 (7)	Sprague Dawley[1]	Male, <200g	Tocris, 25mg/kg (CMC)	**4+3**	73 ± 2	No	RSVP monitoring by telemetry.
Abe et al. Circulation, 2010 (5)	Sprague Dawley[2]	Male, 180-220g	Brand not reported, 20mg/kg (solvent ?)	3+2, 3+5, **3+10.5**	96 ± 11	No	Plexiform lesions at 3+10.5 weeks indistinguishable from human plexiform lesions.

Table. ***Sugen-hypoxia rat models of pulmonary arterial hypertension (2010-2019).*** The rat model consists of a one-time subcutaneous injection of Su5416 (Sugen), followed by a period of 3-5 weeks of hypoxia (10% oxygen). Subsequently, the rats were returned to room air (normoxia) for 0 to 11 weeks (for example, 3 + 6 weeks means 3 weeks hypoxia followed by 6 weeks normoxia. The reported RVSP and quantitative tissue analysis was obtained at the last time point, labeled in bold in the 5th column. In Kojonazarov et al., Wistar Kyoto rats were obtained from the vendor Janvier Labs, Germany. [1]In these studies, male Sprague-Dawley rats were obtained from the vendor Charles River, Sulzfeld, Germany. [2]In these studies, male Sprague-Dawley rats were obtained from the vendor Harlan Laboratories, Indianapolis, Indiana, USA. Abbreviations: CMC, carboxymethylcellulose; ConNx, naïve (no vehicle) normoxic control; CT, computed tomography of the chest; MLI, mean linear intercept, a surrogate parameter for intraalveolar space and emphysema; MCT, monocrotaline; n.s., not significat; PA, pulmonary artery; PAH, pulmonary arterial hypertension; PASP, pulmonary arterial systolic pressure; RV, right ventricle; LV, left ventricle; RVSP, right ventricular systolic pressure; RVH, right ventricular hypertrophy; SuHx, Sugen Hypoxia.

References

1. Bonnet S, Provencher S, Guignabert C, Perros F, Boucherat O, Schermuly RT, Hassoun PM, Rabinovitch M, Nicolls MR, Humbert M. Translating research into improved patient care in pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2017; 195: 583-595.
2. Kojonazarov B, Hadzic S, Ghofrani HA, Grimminger F, Seeger W, Weissmann N, Schermuly RT. Severe emphysema in the SU5416/hypoxia rat model of pulmonary hypertension. *Am J Respir Crit Care Med* 2019.
3. Kasahara Y, Tuder RM, Taraseviciene-Stewart L, Le Cras TD, Abman S, Hirth PK, Waltenberger J, Voelkel NF. Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J Clin Invest* 2000; 106: 1311-1319.
4. Taraseviciene-Stewart L, Kasahara Y, Alger L, Hirth P, Mc Mahon G, Waltenberger J, Voelkel NF, Tuder RM. Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. *FASEB J* 2001; 15: 427-438.
5. Abe K, Toba M, Alzoubi A, Ito M, Fagan KA, Cool CD, Voelkel NF, McMurtry IF, Oka M. Formation of plexiform lesions in experimental severe pulmonary arterial hypertension. *Circulation* 2010; 121: 2747-2754.
6. Legchenko E, Chouvarine P, Borchert P, Fernandez-Gonzalez A, Snay E, Meier M, Maegel L, Mitsialis SA, Rog-Zielinska EA, Kourembanas S, Jonigk D, Hansmann G. The PPARgamma agonist pioglitazone reverses pulmonary hypertension and prevents right heart failure via fatty acid oxidation. *Science Translational Medicine* 2018; 10: eaao0303
7. de Raaf MA, Schalij I, Gomez-Arroyo J, Rol N, Happe C, de Man FS, Vonk-Noordegraaf A, Westerhof N, Voelkel NF, Bogaard HJ. SuHx rat model: partly reversible pulmonary hypertension and progressive intima obstruction. *Eur Respir J* 2014; 44: 160-168.
8. Jiang B, Deng Y, Suen C, Taha M, Chaudhary KR, Courtman DW, Stewart DJ. Marked strain-specific differences in the SU5416 rat model of severe pulmonary arterial hypertension. *Am J Respir Cell Mol Biol* 2016; 54: 461-468.
9. Dean A, Gregorc T, Docherty CK, Harvey KY, Nilsen M, Morrell NW, MacLean MR. Role of the Aryl Hydrocarbon Receptor in Sugen 5416-induced Experimental Pulmonary Hypertension. *Am J Respir Cell Mol Biol* 2018; 58: 320-330.
10. Dabral S, Tian X, Kojonazarov B, Savai R, Ghofrani HA, Weissmann N, Florio M, Sun J, Jonigk D, Maegel L, Grimminger F, Seeger W, Savai Pullamsetti S, Schermuly RT. Notch1 signalling regulates endothelial proliferation and apoptosis in pulmonary arterial hypertension. *Eur Respir J* 2016; 48: 1137-1149.

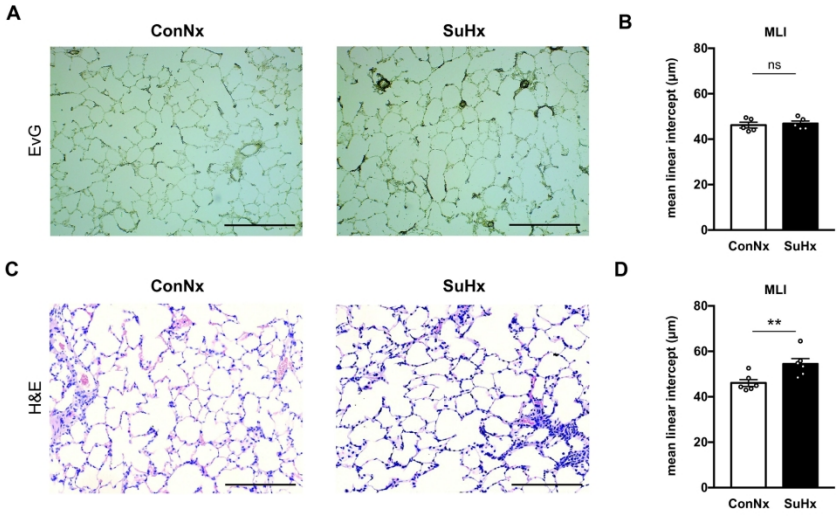

Figure 1

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