Medical Interventions to Reverse Pulmonary Hypoplasia in the Animal Model of Congenital Diaphragmatic Hernia: A Systematic Review

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Abstract:

Objectives: We aimed to systematically review all published pre-clinical research on prenatal medical treatment of pulmonary hypoplasia in CDH.

Background: The neonatal mortality due to isolated congenital diaphragmatic hernia (CDH) remains high. Whether fetal endoscopic tracheal occlusion (FETO) reduces mortality is still to be demonstrated. Therefore more potent preferentially medical therapy would be welcomed.

Methods: We searched MEDLINE (Pubmed), Embase and the Web of Science including all studies from the earliest date (1951) to December 2013. Article quality was assessed using the modified CAMRADES checklist. Inclusion criteria were those animal studies addressing prenatal medical interventions and principal variables were confirmation of a diaphragmatic defect, lung to body weight ratio (LBWR), formal airway morphometry or DNA/protein content.

Results: In total 983 articles were identified. Following abstract review, 96 articles were assessed by two authors in agreement with a third for eligibility. Of these, 43 were included in the final analysis. The median number of study quality checklist items (maximum 10) scored was 4 (IQ range: 2-5). Twenty-seven (69.8%) of studies were in the nitrofen rat. The majority were treated with vitamins or glucocorticoids. Single studies reported some improvement in lung morphology with alternative therapies. It was impossible to identify a pattern in animal model selection or creation, mode, time point or duration of treatment and readouts. Only one study reported a sample size calculation.

51 **Conclusion:** Comparison in pre-clinical studies in CDH is challenging due to methodological
52 variation. Agreed standardized methods need to be applied in future investigation of new
53 medical therapies.

54

55 **Introduction**

56 Congenital diaphragmatic hernia (CDH) is a relatively common condition of the fetus occurring
57 in 1 in 2500 live births(Langham et al. 1996). The diaphragmatic defect results in herniation
58 of the viscera during the embryonic phase of pulmonary development, competing for space
59 in the thorax with the growing lungs. Affected lungs demonstrate pulmonary hypoplasia (PH)
60 with fewer distal airways, reduced size and number of alveoli with thickened alveolar walls
61 and an increased volume of interstitial tissue(Askenazi and Perlman 1979)(AREECHON and EID
62 1963). Over 60% of CDH cases are diagnosed by the second trimester of pregnancy on
63 screening ultrasound (Garne et al. 2002) of which some are eligible for fetal therapy. Fetal
64 Endoscopic Tracheal Occlusion (FETO) involving placement of a balloon to occlude the fetal
65 trachea, prevents egress of lung fluid, leading to increased pulmonary stretch(Khan, Cloutier,
66 and Piedboeuf 2007). This increases airway branching morphogenesis and promotes
67 pulmonary vasculature maturation (DiFiore et al. 1994)(Jani et al. 2009)(Deprest, Nicolaides,
68 and Gratacos 2011). The definitive conclusion on whether FETO improves survival is awaited
69 from the multi-center randomized control trial TOTAL (tracheal occlusion to accelerate lung
70 growth)(Dekoninck et al. 2011). Regardless, FETO carries significant risks including pre-term
71 rupture of membranes (47.1%) with delivery before 34 weeks in 30.9% or before 32 weeks in
72 17.1%. Other complications include chorio-amnionitis (2.4%) and neonatal mortality and
73 tracheal morbidity due to difficulties with balloon removal (4.8%). With survival reaching 50%
74 at best ultimately new treatments need to be pioneered(Jani et al. 2009).

75 Postnatal outcome can be predicted by the severity of antenatal PH which leads to respiratory
76 insufficiency in the neonate (Deprest et al. 2009). Persistent pulmonary hypertension of the
77 newborn (PPHN) is a second major factor determining outcome that lacks adequate

treatment(Thébaud, Mercier, and Dinh-Xuan 1998). This is due to a combination of pre-natal pulmonary vascular hypoplasia with vascular remodeling and altered pulmonary artery responsiveness to various vaso-active mediators in the CDH lung(Dillon et al. 2004)(T Okazaki et al. 1997)(Tadaharu Okazaki et al. 2008)(T Okazaki et al. 1998). These unresolved factors have resulted in an unacceptable mortality rate despite in utero referral to a high volume center offering standardized neonatal care (van Loenhout et al. 2009).

There are several well-established animal models of CDH whose lung development mimics the pulmonary hypoplasia seen in the human condition(Pringle et al. 1984). This review will focus on those models which have been most widely investigated in pre-natal intervention, namely the nitrofen (NF) rat, the rabbit and sheep. The herbicide NF if given by gastric gavage at a specific gestational age in pregnant rats induces diaphragmatic hernia in up to 50% of fetuses(Allan and Greer 2012). This model has the advantage that there is a resulting independent lung and diaphragmatic defect in the embryological stage of lung development mimicking the “dual-hit” seen in humans(Keijzer et al. 2000). The creation of a diaphragmatic defect in the rabbit and lamb is most commonly during the pseudoglandular stage of lung development(Wu 2000)(Pringle et al. 1984). Although pulmonary hypoplasia is directly secondary to the surgical defect, rabbits and sheep have the advantage that their lung development is more similar to humans; unlike in rats where they alveolize prior to birth(Pringle 1986)(Iwasa et al. 2011)(Yamada et al. 2002).The aim of this systematic review is to identify published pre-clinical research on the medical treatment of PH in the setting of congenital diaphragmatic hernia.

METHODS

This systematic review was structured in line with the previous guidance provided in the PRISMA statement (Preferred Reporting Items for Systematic reviews and Meta-analyses)(Moher et al. 2010).

Literature search

A complete computerized literature search was conducted using MEDLINE (Pubmed), Embase and the Web of Science including all studies from the earliest date (1951) to December 2014. The electronic search strategy included both Medical Subject Headings (MeSH) and keywords (Appendix 1). Mendeley was used to eliminate duplicate reports. Reference lists and topic-related reviews were checked manually to identify further relevant papers.

Inclusion and exclusion criteria

Studies taking place in animal models of CDH undergoing antenatal treatment to reverse pulmonary hypoplasia preferably with a confirmed diaphragmatic defect were included. In case of missing information, corresponding authors were contacted and asked to provide additional data. Outcomes measures included reporting of lung to body weight ratio (LBWR), formal airway morphometry or an alternative method of airway morphology (radial alveolar count (RAC)). Studies reporting only vascular morphometry were excluded. Control groups were also documented. Letters, in vitro studies, review articles and abstracts were also excluded.

Selection of Studies

Study titles and abstracts were screened by one author (MPE) to identify the relevant articles. Foreign language articles were included. Relevant articles were independently reviewed by

two authors (MPE/ FMR) according to the inclusion criteria, quality assessment and risk of bias (Appendix 2). Disagreements between the reviewers were resolved by discussion with the third author (JD). The Kappa between the first 2 authors and then with the third author was calculated to determine the strength of agreement. In case of overlapping studies, only the largest and most complete data set was included.

Quality Appraisal

Study quality and risk of bias were assessed using a checklist adapted from the Cochrane handbook for systematic reviews of interventions, the good laboratory practice guidelines for in vivo stroke modeling and the Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES) quality checklist (Appendix 2)(“CAMRADES”; Macleod et al. 2009)(Pedder et al. 2014).

Data Extraction

A pre-designed form was used for independent data extraction by two authors (MPE/FMR) (Appendix 2). Data ~~were~~^{was} extracted on the authors, the animal species and gestational day (GD) of diaphragmatic hernia induction with information on the type, GD, route and duration of treatment noted. Outcome measures were recorded as noted in inclusion criteria.

RESULTS

Literature Search

Our literature search identified ~~982302~~ publications (Figure 1). Of these ~~88640~~ were excluded on reading of the title and abstract; case report or review (n=~~26634~~), human trial (n=~~16132~~), intervention was tracheal occlusion only (n=~~712~~), other disease model (n=~~10098~~), not an antenatal medical therapy (n=~~2362~~) or reporting of an in-vitro study (n=~~524~~). This left ~~954~~ studies for further full-text evaluation (Figure 1). ~~Nineteen~~ ~~Twenty-seven~~ of these, the majority from one group (Puri, Dublin) were analyzed as a subgroup (table 5+6). This was due to these studies focusing on the regulation of target genes, transcription factors or proteins in response to antenatal treatment with dexamethasone, retinoic acid or a TRH rather than on pulmonary outcome measures. A further ~~3427~~ publications were excluded on full text review as they reported no relevant pulmonary outcome measures (n=~~2318~~), data ~~were~~ reported elsewhere (n=1), not a CDH model (n=4). Ten papers required discussion with the third author (JD) prior to exclusion. The Kappa between the first 2 authors was calculated at 0.872. Discussion with a 3rd author JD produced a Kappa of 0.765. Papers on experiments with tracheal occlusions alongside ante-natally medical therapy were included with groups of animals who did not undergo tracheal occlusion.

Study quality

The median number of study quality checklist items (maximum 10) scored was 4 (range: 2-7) (Table 1). All studies were published in peer-reviewed journals, ~~only~~ none described a sample size calculation ~~with 3/43 (7.0%) and only one document~~ing control of temperature. ~~Nineteen-Twenty~~ (46.5/48.7%) reported random allocation to treatment group, none reported blinding to group allocation during animal treatment, 9/4339 (20.9/3.1%) reported blinded assessment to outcome (airway morphometry), 11/438/39 (25.6/20.5%) included a statement regarding conflicts of interest, 363/4339 (83.7/4.6%) stated compliance with local animal welfare regulations (Table 1).

Medical therapies

In total 3027 (69.8/69.2%) studies were completed in the NF rat (Table 2), 76 (16.2/15.4%) in the rabbit (Table 3) and 6 (14.0/15.4%) in the lamb (Table 4). These were further divided into treatments as shown in figure 2. There were no therapeutic studies identified in the mouse model.

Creation of Controls

In the NF rat model control groups existed at two time-points; the creation of the model using NF vehicle (olive-oil) and later at the time of fetal treatment. Nineteen studies (70.4%) included olive oil without NF as a control group. There were no uncontrolled studies. In rabbits, there were no studies including sham controls at the creation of the model level. All studies included “internal” normal controls being littermates. In lamb studies, only 1/6 studies undertook sham creation of CDH; the other studies used instead un-operated internal or external controls.

Creation of Diaphragmatic Hernia

The majority of rats (7~~6.74~~⁷⁵%) had NF administered on E9.5 and in the remaining 7 studies NF was given E8.5-13. Of these 2~~75~~⁷⁵/~~3027~~³⁰²⁷ (9~~03~~⁰³%) studies used Sprague-Dawley rats the remaining ~~32~~³² studies using the Wistar strain (Mayer et al. 2008)(Baptista et al. 2005). In rabbits, creation of DH was either d23 (n=~~32~~³²), d24 (n=2) or d25 (n=2) of gestation. In sheep DH creation ranged from GD 65 to 85 (term = 145 GD) from mid-pseudoglandular to early canalicular stage of lung development.

Routes of Treatment

The majority of rat experiments employed transplacental delivery of substances to the fetuses. In six studies (~~2017.6~~^{2017.6}%) dams received two medical therapies or a medical therapy and TO (2) (Table 2). Mothers were medicated intra-peritoneally (IP) (~~4038.2~~^{4038.2}%), by gavage (~~23.30.5~~^{23.30.5}%), subcutaneously (SC) (~~23.30.5~~^{23.30.5}%), or with a SC/IP implanted pump (~~2014.7~~^{2014.7}%). Two (~~6.75.9~~^{6.75.9}%) papers described direct fetal administration with intra-amniotic injection. In rabbits (~~76~~⁷⁶ studies), 2 studies used a maternal intramuscular injection, ~~32~~³² studies employed a gel plug or PFOB in the fetal trachea, and another two studies used the fetal amniotic route.

In the sheep model half of the studies involved transplacental medical therapy using the maternal intra-muscular (IM), intra-venous (IV), or peri-oral route. In 3 (50%) studies it involved direct fetal therapy: two were in the chronic instrumented lamb (fetal IV administration), and in one intra-tracheal injection was used.

Results per medical strategy

Due to the lack of an identifiable pattern each treatment is broadly classified by route, treatment regime, species and outcome.

Vitamin A (Vit A)/ Retinoic Acid (RA)

Five rat (table 2), 1 rabbit (table 3) and 1 lamb (table 4) paper studied RA or Vit A as prenatal treatment in the CDH model. In rats, route, GD of start (E9.5-E20.5) and duration (1-5 days) of treatment was variable. Rabbits had daily IM RA for 7 days in late gestation, in lambs 2 weeks of IV fetal treatment was given again in late gestation(Gallot et al. 2008). In rats, early administration of Vit A (< E 14) improved LBWR in 3 (60%) studies however late administration ($\geq$ E16) had no impact. However there was some suggestion of a significant improvement in vascular indices(Schmidt et al. 2012). Thebaud et al showed Radial Saccular Count (RSC) to be improved to control levels with early IP Vitamin A(Thébaud et al. 1999). In the rabbit model RA IM for 7 days had no effect on lung morphometry, there was however an increase in surfactant protein B (SP-B) levels(Gallot et al. 2008). Vit A in late gestation sheep significantly improved LBWR, lung compliance and reported improvement in histology yet without any quantitative measures being detailed (Lewis et al. 2011).

Other Vitamins

In the NF rat model studies employing treatment with vitamin C (2 papers) and, E (5 papers) and NAC (1 paper) were identified (table 3). Vit C was administered IP daily from E16 until E20 with a reported improvement in SPB levels and histology but no increase in lung total protein levels or LBWR(González-Reyes, Martínez, and Tovar 2005)(Cigdem et al. 2010). In five studies Vit E was given, in 3 by gavage, the other 2 either IP or SC. Four (80%) treated once daily E16-20, in the final paper were Vit E was given SC on E11. Islam et al showed significant improvement in LBWR ratio with Vit E gavage, however this was not confirmed in two later studies(Islam et al. 1999; Salome Gonzalez-Reyes, Martinez, et al. 2006; González-Reyes,

Martínez, and Tovar 2005). Although several studies repeated increased lung protein/ DNA content, histology was not significantly improved in any study (table 2).

Steroids

Steroids, namely beclomethasone (BPD) (n=3), dexamethasone (dex) (n=~~6~~5) or prednisolone (pred) (n=1) were administered in ~~4~~3 rat, 3 rabbit and 3 sheep studies. Route and medication dose varied greatly between studies. In rats, 2/~~4~~3 studies employed TO concurrent to steroids at E18.5-19 of gestation. Steroid administration was always on E18.5-19.5 once IP ~~or daily IP~~ and a third it was given SC daily. There was no significant improvement in LBWR in any study, with ~~two~~one study~~ie~~ reporting an improvement in airway histology (increased sacular volume)(Suen, Bloch, and Donahoe 1994)(Gonçalves et al. 2014). Steroids upregulated VEGF-r expression but failed to upregulate mRNA SPA and SPB in single studies(Schmidt et al. 2010; Suen, Bloch, and Donahoe 1994)(Gonçalves et al. 2014). In rabbits 2 studies delivered steroids IA, immediately following CDH creation. This was reported to show improvement in histology (alveolar and septal density) in one study without increase in LBWR(Tannuri et al. 1998). The third study in rabbits delivered IM steroids GD29 with and without TO. This had no impact on LBWR but did improve vascular morphometry, increasing the numbers of small vessels and reducing the muscularisation of peripheral lung vessels more significantly than TO alone(Roubliova et al. 2010). In sheep 2 studies delivered IV fetal steroids and the third IM to the ewe late in gestation. Although GC had no additive affect to TO, there was an improvement in airway morphometry in 3 studies (table 5).

Steroids +Thyrotropin Releasing Hormone (TRH)

Four additional studies with steroids and TRH were undertaken in the NF rat (table 2). Steroids (IP) alongside an implanted IP pump to deliver TRH continuously were administered E18.5-

19.5. No study showed an improvement in LBWR, some reported an improvement in histology, in particular radial alveolar ~~(RAC)~~ count (RAC) however TRH alone had no impact(Suen et al. 1994). TRH was not tested in larger animal models.

Isolated Studies

Multiple other therapies in rats have been attempted (table 2). Of note tetrandrine (2 studies) improved LBWR and morphology (RAC)(Lin et al. 2007; Xu, Liu, Wang, et al. 2009). GLP-1 restored LBWR; with the authors reporting improvement in airway morphometry and SP-C levels in NF rats, however without any quantitative measurements(Romaní-Pérez et al. 2013). The endothelin inhibitor (CGS 26303) in the NF-rat significantly improved LBWR with a two fold increase in postnatal survival in CDH fetuses [58]. Sildenafil, although did not improve LBWR, yet it improved airway (Lm) and vascular indices in the NF rat. It reduced arterial wall thickness, right ventricular hypertrophy, increasing pulmonary vessel density and pulmonary artery relaxation(Luong et al. 2011). The angiotension-2 antagonist (AT2) improved LBWR significantly with an improvement in RSC, the pulmonary artery was also less muscularized (Nogueira-Silva et al. 2012). Other work using amniotic fluid stem cells (SC), administered intra-amniotically to the NF-rat in early gestation and resulted in a restored terminal bud count in hypoplastic lung explants[52]. More recently human lung SC have been shown to promote lung maturation(Yuniartha et al. 2014). Multiple other therapies including (table 3) ghrelin, estrogen, bombesin, cystic fibrosis transmembrane regulator gene therapy (CFTR) and epidermal growth factor (EGF) in the NF-rat have again improved yet failed to normalize LBWR[53][54][55][56][57](Sakai et al. 2014).

Two studies in rabbits (table 3) employed an IT fibrin gel plug on GD27, both improving LBWR but not to the levels of normal suture ligation(Elattal et al. 2013)(Muensterer et al. 2012). Also

there was improvement in pulmonary function tests, airway (RAC) or vascular morphometry (reduced vessel thickness) compared to controls. Recently, it was shown that IT preflurooctylbromide (PFOB) also improved lung mechanics(Herber-Jonat et al. 2014). In lambs, ~~(table 4)~~ maternal PO tadalafil (phosphodiesterase inhibitor) improved fetal pulmonary blood flow but had no impact on LBWR (table 4) (Shue et al. 2014). A second study in the lamb model delivered a single dose of KGF IT which had little impact on RAC or LBWR(Saada et al. 2010).

Subgroup Analysis

~~Nineteen~~^{Twenty} papers that were published between 1998 and 2001, the majority from Puri et al (Dublin), investigate the molecular biology of the NF rat CDH lung (Table 5+6). They focus on the mechanisms by which antenatal administration of either RA or dexamethasone (dex) work to accelerate lung development. Antenatal therapy of dex or RA was administered to the dam once a day IP in late gestation (Table 5+6). One study also included an infusion with TRH and looked at the expression of SP-B and TTF-1, resulting in a ~~*5-fold~~ up-regulation of TTF-1 levels and ~~*10-fold~~ increase in SP-B levels towards the control(Losada et al. 2000). Specific gene and protein expression where measured in each group, firstly by PCR and protein expression was confirmed either by immunohistochemistry, ELISA or by appropriate assay (Table 5+6). Significant results in the dex treated NF rat included significant up-regulation of bFGF, eNOS, and total RNA content towards controls. With significant down-regulation in treated NF rats of PDGF-A, and TNF- α again toward controls. In NF rats treated with RA there was significant up-regulation of RAR α , IGF-2, Cx 43, IGF-1R/2R, LGL1, CTCR, MK, PTHrP-R, P13K, AKT and ICAM-1 towards controls. TTF-1 was significantly down-regulated in treated NF rats.

Differences in Airway Morphometry

Formal airway reporting employing measures such as RAC, RSC, Linear intercept (Lm) and mean terminal bronchial density (MTBD) was included in ~~120~~ (40.37.0%) rat, ~~21~~ (28.616.6%) rabbit and 3 (50%) sheep studies. The use of pressure fixation of lungs prior to airway morphometry was not uniform. In total, ~~12 eleven~~ (70.68.5%) studies pressure fixed their lungs, 8 rat studies pressure fixed their lungs (50% for 24hours, rest for 48 hours); ~~both~~ rabbit studies (24 hours), 2 lamb studies (24 hours and 1 week).

Discussion

We are not aware of any previous systematic review to compare pre-clinical treatments for lung hypoplasia in CDH. Primarily, pre-clinical treatments have focused on steroids and vitamins. The former were shown to be effective both in the rodent(Suen, Bloch, and Donahoe 1994), rabbit(Roubliova et al. 2009) and in ovine model(Hedrick et al. 1997), yet lambs were delivered slightly prematurely (136/145 days). A randomized controlled clinical trial in 32 mothers pregnant with CDH fetuses using two doses of betamethasone (12.5mg) or placebo 24 hours apart, weekly from 34 to 36 weeks failed to show any benefit in survival or morbidity in steroid exposed fetuses (Lally et al. 2006). Targeting the retinoic acid pathway using vitamin A or its analogs is effective in rodents and in one study in lambs though judged not clinically possible due to concerns over teratogenic effects (Tzimas and Nau 2001). In the absence of any effective or realistic medical therapy, it remains an important area of research. To standardize and allow comparison between future experiments methodology needs to be more standardized. Multiple levels of variation exist from the outset of these experiments to the reporting measurements.

The first level of variation is in selection of the animal model. The NF rat with its “dual-hit” affecting both lung and diaphragmatic development in the embryonic period independently

is an attractive model(Keijzer et al. 2000). However, NF is given at varying gestational time-points, therefore producing a range of diaphragmatic and lung defects making comparison difficult(Kling and Schnitzer 2007)(Kluth et al. 1990). Interestingly the CDH varies between the rat strain even with NF administered on the same gestational day; Sprague Dawley (59%), Long Evans Hooded (18%) and Wistar (37%)(Kling and Schnitzer 2007)(Kang, Zolna, and Manson 1986)(Mayer et al. 2008). In larger animal models such as the sheep and rabbit, the diaphragmatic defect is surgically created, at the earliest in the pseudoglandular phase for technical reasons (Wu 2000)(Kent et al. 1972). This introduces two variables; defect size and time point of induction(Langwieler et al. 2004). Thoracotomy in fetal rabbits has been done as early as GD20 but there was no survival at this time point(Fauza et al. 1994). Fauza et al also demonstrated that induction at GD 24 or 25 leads to a significant decrease of alveolar intercept with a 30% fetal loss rate (Fauza et al. 1994). Wu et al later reported an 87% fetal survival on rabbits operated on GD23 (Wu 2000). In sheep, DH was in the vast majority created between GD 65-85; with later induction leading to less prominent hypoplasia. Apart from a difference in the time point DH is induced, the models also differ in the stage of lung development the pups are ultimately born. Sheep are established in the alveolar phase at term, while rats are in the saccular phase(“Pringle et Al 1986.pdf”). Humans similar to rabbits, are ~~at term~~-born at term in the early alveolar phase of lung development and unlike rats who only alveolarize from 4 days post-natally(“Pringle et Al 1986.pdf”). In summary, each model carries its individual merit and there is a valid argument ~~with it necessary for treatments which are-for~~ successful treatments in NF rat to be tested in a larger animal model before moving into clinical trials.

Treatment route is another important variant. Clinically, the preferred route is trans-placental which is less invasive and allows for repeated dosing. In the majority (62.815~~5~~%) of the animal

studies the trans-placental route (oral, IP, IV or SC administration) was used. The medical therapy can also be administered directly to the fetus, either non- or minimally invasively (from the fetal viewpoint) percutaneously, or invasively by fetal injection. Fetal breathing movements commence at 10 weeks in humans, in lambs at GD 40, in rabbits at GD 25 and in rats at GD 16 (Dawes et al. 1972) (Galan et al. 1997)(de Vries and Fong 2006)(Kobayashi, Lemke, and Greer 2001). Though repeated intra amniotic injection would be feasible, in smaller animals it becomes impractical, because it requires anesthetic and most studies also use laparotomy. [9].

The third level of complexity is the variability in readout measures between animal models and individual studies. Species specific readouts are necessary when considering these models, not least because of the variation in stages of lung development at term between models as previously mentioned("Pringle et Al 1986.pdf"). LBWR is a pathological measurement but in the studies we identified it was not universally reported (87.6%). The cut-off LBWR for PH was defined by Wigglesworth at $\leq 1.5\%$ at < 28 weeks and $\leq 1.2\%$ at ≥ 28 weeks gestation(Wigglesworth and Desai 1981). Between NF rat studies we could not identify an agreed consensus regarding LBWR. For instance, in the study by Luong et al on NF-rats, the LBWR at term was 2.4% in CDH fetuses vs. 3.1 % in olive oil exposed fetuses. In the study by Santos et al, CDH fetuses had a LBWR of 1.7% as opposed to 2.2% in controls(Baird et al. 2008). Both studies fed olive oil to controls on E9.5, used Sprague Dawley rats which were harvested at E21.5. However, in the latter study controls received a SC placebo injection from E13.5-17.5, in the first study dams remained untouched after E9.5. We could not identify any other differences. These discrepancies demonstrate the need of appropriate control groups when reporting data. In rabbits and indeed sheep the impact of the time point at which the diaphragm is partially resected becomes very obvious. In one of the earlier studies in lambs,

Starrett and de Lorimier created DH at GD 127-144 leading to a reduction in LBWR from 2.9% to 2.2% whereas Shue operating at GD 75 had a LBWR reduction from 3.4 to 1.8% in CDH fetuses(Starrett and de Lorimier 1975)(Shue et al. 2014). Lung hypoplasia should also be confirmed by assessment of the DNA/protein content to ensure the change in weight due to medical intervention is not secondary to tissue edema in the airways (Wigglesworth and Desai 1981). Similarly, using dry lung weights discounts the effect of fluid retention(Davey et al. 2006). Conversely, therapy effects may be quantified ~~by~~ proliferation stain indicating additional lung growth compared to untreated CDH controls(Mayer et al. 2008)(Salome Gonzalez-Reyes, Fernandez-Dumont, et al. 2006).

Morphology of the lung is another level of quantifying lung development. The American Thoracic and European Respiratory Society have published clear guidelines on the quantitative assessment of airway structure for basic and translational lung research in 2010(Hsia et al. 2010). They suggest~~ed~~ that fixation should be ideally with 2.5% glutaraldehyde or alternatively formaldehyde at a pressure of 20-25cm H₂O for at least 24 hours where after specimens should be dehydrated in ethanol. In this review we are able to report formal histology with pressure fixation in a small number (28.5%), with it being unclear in many studies whether fetuses were permitted to breath before fixation. However, one study comparing morphometry between pressure fixed lungs and fetal lungs that never respired with no large differences found. Roubliova speculated that this was due to the lack of alveolized parenchyma in hypoplasia CDH lungs (Roubliova et al. 2010). For optimal morphometry, systematic random uniform sampling (SURS) is recommended, with the initial random cut through the lung followed by serial parallel slices at a constant thickness interval(Hsia et al. 2010). As far as we could see, these techniques have not been employed in animal studies. However, the small sizes of rat and rabbit lungs allow coronal sections through the entire lung

to be taken on each slice. In summary, we identified at least four variables that could influence findings (duration, substance and pressure for fixation, and whether or not the animal is allowed to respire), leading to difficulties in comparison of outcomes between studies.

This systematic review emphasizes the need for the standardization of studies in CDH animal models; in the timing of CDH creation, the treatment of specimens and reporting measures.

We have several suggestions following to reduce the degree of variability at each level in these studies. At the model creation level, one could argue for administration of NF on E9-9.5 and using Sprague-Dawley rats due to their sensitivity to NF and the predominance of LCDH on that day. Also, only lungs from animals with a confirmed defect should be included as animals without may not have truly hypoplastic lungs. Surgical creation in rabbits seems most successful in terms of balancing effect and fetal survival at GD23. In lambs surgical creation should be done as early as GD65-80 to obtain true hypoplasia. For planning numbers of animals in experiments authors should report fetal loss rates. At the reporting level, LBWR should be reported. Most studies are done with pressure fixation, using a pressure and a duration which is fitting for the tissue volume. Following fixation lung some recognized quantification tool for lung morphometry should be employed whether it is RAC, RSC, Lm, LMW or MTBD. Employing these measures will allow a fairer comparison between treatment modalities as more strategies emerge.

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