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Reproductive Performance and Factors Influencing Fetal Outcome: A Practical Guide to Experiments on Lung Development in A Nitrofen-Induced Rat Model for Congenital Diaphragmatic Hernia

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Abstract

Our study aims to help researchers calculate resources based on a rat breeding colony for experiments on fetal lung development.

The Wistar rat is commonly used in experimental research. In the context of fetal lung studies, the nitrofen rat is a model for pulmonary hypoplasia and congenital diaphragmatic hernia. Data needed to calculate resources for new experiments are not easily available.

We prospectively acquired data on the reproductive performance and fetal outcome of 314 consecutive virgin Wistar dams in our fetal lung research breeding colony.

We define the impact of breeding conditions on rat fertility and evaluate different methods to diagnose early rat gestation. Effects of nitrofen exposure as well as fetal surgery in terms of prenatal mortality and gross anatomical parameters of lung development are quantified in nitrofen-exposed and -unexposed fetuses from embryonic day 19 to 21 in 12h intervals.

A reduced mating interval (1h) provides a great degree of experimental control with feasible pregnancy rates and a large litter size. Nitrofen exposure, as well as fetal surgery, depict high fetal survival rates. Fetal lung findings were very reproducible and could allow for reduction of animals utilized within experimental groups.

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Introduction

Rats reach estrous approximately every fourth day.[1] A fixed regimen of light and dark intervals is essential to establish fertile cycles which can be monitored by vaginal smears.[2] A typical age for breeding rats is around 12 weeks.[3] Light-dark cycle, parental age, parity, ambient temperature as well as diet were shown to impact rat fertility.[4-7] Pregnancy is indicated by a vaginal plug, sperm in a vaginal smear, subsequent maternal weight gain and later by direct palpation. Manipulations may induce stress responses as well as pseudopregnancy, the later being revealed by a persistent abundance of mucus in vaginal smears.[8] Ultrasound was suggested but so far has not been proven to be a simple and reliable device in detecting early rat gestation.[9]

A setting in which early diagnosis of pregnancy is desired is before administering nitrofen (NF) to any dam. First reports about the teratogenic potential of this herbicide were published in 1971.[10] 20 years later Kluth et al. suggested the "NF rat" as a model for pulmonary hypoplasia and congenital diaphragmatic hernia (CDH).[11]

CDH occurs in 1-2/5,000 newborns. At birth, the associated lung hypoplasia leads to pulmonary insufficiency and hypertension, which is fatal in up to 30% of babies in whom the condition is isolated.[12,13] Severe pulmonary hypoplasia can be diagnosed prior to birth defining a subset of fetuses that may benefit from prenatal interventions. Fetal tracheal occlusion (TO) prevents the egress of pulmonary fluid thereby increasing tissue stretch and accelerating lung growth.[14] It is offered in experimental settings with the major risk of preterm delivery. Optimal timing has not yet been defined.[15] Studies in animal models such as the NF rat are ongoing and an essential part in the process of understanding the underlying pathophysiology of lung hypoplasia as well as the effects of any antenatal intervention: Typically 100mg NF is administered orally to pregnant dams on embryonic day (ED) 9 or ≥ED10 to induce fetal left (LCDH) or, respectively, right CDH (RCDH).[11] Studies using this model differ greatly in terms of conditions as e.g. rat strain, mode of NF application and percentage as well as the type of CDH. It is not always clear how these differences can be explained.[11,16] Often the experimental details in these papers are scarce, methods are sometimes outdated and unanticipated confounders are still a concern.[3,17] Given the clinical relevance of TO timing and the short rat gestation substantial changes in fetal lung development occur within hours.[18] Researchers hence might want to control their experiments from the outset, i.e. before rat copulation instead of ordering time-mated rats. In this novel and comprehensive study, we share our experience with a 1h-mating protocol in 314 Wistar dams in order to equip new investigators with sufficient information to plan and power experiments in the field of fetal lung research.

Experimental Procedure, Materials and Methods

Animals and Housing Conditions

This is a prospective analysis of the reproductive performance and fetal outcome of 314 consecutive virgin Wistar dams which were part of our experiments on prenatal lung development. Female rats were ordered at the age of 9-14 weeks, male rats (n=20) at the age of 11 weeks (Elevage Janvier, Le Genest-St-Isle, France). All were first given one week to adjust to their new environment. Animals were accommodated in a separate room of the animalium of the Faculty of Medicine, KU Leuven. This room was only accessible to the staff and the researchers directly involved in animal care (V. B., S. D., T. D.). The top rows of the shelves were avoided for a possible negative effect of direct light.[1] Males were housed alone, females in pairs (type III cage: ground area 810cm^2, height 19cm) with free access to food (V1535-000 R/M-H 15mm, Ssniff Spezialdiäten, Soest, Germany) and water. Bedding consisted of wood fibers (Lignocel Hygienic Animal Bedding, J. Rettenmaier & Söhne, Rosenberg, Germany) and cage enrichment was provided in form of paper towels after each handling. The day-night cycle comprised a 14-h-light followed by a 10-h-dark period (21.30–7.30). Temperature was pre-set to 20°C and cages were changed twice per week (Monday and Thursday).

Reproductive Performance

To determine the reproductive cycle in female rats, vaginal smears were obtained over at least 4 consecutive days (12.00-14.00). These were analyzed by light microscopy (Zeiss Axioplan microscope, Carl

Zeiss, Oberkochen, Germany).[2,19] The number of pseudopregnancies induced by repeated vaginal smears as detected by highly mucous vaginal secretion was noted.[8] The established cycle was confirmed as pro-estrous around noon before the scheduled mating. At that time, the cage of the selected female was arranged next to that of the male, in such way that they could see and smell each other, however, without any possible physical contact for 9h (14:00-23:00). Mating was then allowed by putting the female into the cage of the respective male for 1h (23:00-0:00). Just prior to that, the female rat was weighed (N° 8.589, Berkel, Brussels, Belgium measuring accurately up to 5g). Directly after the separation of the male and female rat a vaginal smear was obtained to check microscopically for the presence of sperm. The amount of sperm was recorded as none, single, few, or plenty. Quality was assessed as presence of complete sperm or mere fragments. The presence of a vaginal plug was determined either at this time-point or at the latest early the next morning (≤7:30). This day was defined as ED0 of a potential pregnancy (term=22 days). Non-pregnant status was confirmed by monitoring females for the length of a normal gestation. After that, non-pregnant rats were available for a second/ third mating attempt following the protocol as described above. Additional measures recorded were maternal and paternal age, the date and the number of the mating attempt as well as the mating partner.

Interventions during Pregnancy

Pulmonary hypoplasia and CDH were induced on ED9 (embryonic phase of lung development) by gavage feeding 100mg of NF (dissolved in 1ml of olive oil) (Maybridge, Acros Organics NV, Geel, Belgium) to pregnant rats. (NF was given independently of female weight. It was suggested, that data might be more valid and uniform if NF dosage would be based on actual weight, which could be explored in a future study.) Those animals later underwent fetal surgery, consisting either of TO with a clip or sham surgery with neck dissection only (sham) on ED18, ED18.5 (both pseudoglandular phase), ED19 (pseudoglandular-cannalicular phase) or ED20 (cannalicular phase) as earlier reported in detail.[20] Unoperated fetuses of the same litters served as controls. Fetuses were identified by their position; dead fetuses and fetuses that succumbed during fetal surgery were recorded. NF-unexposed (NF- versus NF+) fetuses served as healthy controls. Non-pregnant rats that had received NF were euthanized.

Harvest of Fetal Lungs

Harvest was performed at 0.5-day intervals between ED18 and ED21. Following laparotomy, the uterus was assessed for the presence and viability of fetuses and compared to the status at fetal surgery. Fetuses were harvested one by one through hysterotomy under general maternal anesthesia. We recorded the fetal body weight (fBW), the fetal right and total lung weight (fRLW, fTLW) (Acculab, Sartorius group, VIC-303, NY measuring accurately up to 0.001g), as well as the presence or absence of CDH (CDH-) [LCDH, RCDH, bilateral CDH (BCDH)]. Fetal left lung weight (fLLW) and fetal lung-body-weight ratio (fLBWR) were calculated from the obtained values. Female weight was obtained before NF-administration, surgery and harvest.

Environmental Conditions

There were two episodes of pinworm infections of other animals housed in the same facility, and our animals received preventive treatment with fenbendazole (FBZ)-medicated pellets (A153F0300 R/M-H 10mm containing 3g/kg Fenbendatat 5%, Ssniff Spezialdiäten; nutritional value identical to regular pellets. Target dose 8-12mg/kg/day for 2 months after diagnosis of the infection[21]). In addition, a general floor and surface disinfection procedure using Clidox-S (application according to the manufacturer's recommendations, Pharmacal research laboratories, Waterbury, CT) was performed daily for two months, in addition to regular hygienic measures.

Statistical Analyses and Ethics Committee Approval

Data are presented as mean +/- standard error of the mean. Maternal weight and parental age is specified as mean +/- standard deviation. Absolute numbers and percentages are given if applicable. Sets on fetal survival rates and biometrical data in the different experimental groups were analyzed by Kruskal-Wallis and Dunn's multiple comparison test (GraphPadPrism, Version 5.0, San Diego, CA). Remaining data was tested by uni-/ multivariate stepwise regression analysis (JMP, Version 7.0, Cary,

NC). P-values <0.05 were considered statistically significant (* <0.05; ** <0.01; *** <0.001). Data on the very early intervention groups are not displayed for easy reading purposes.

The experimental protocol was approved by the Ethics Committee for Animal Experimentation of the Faculty of Medicine, KU Leuven. Rats were treated according to current guidelines on animal well-being.[22,23]

Results

Characteristics of the Adult rat Population and Environmental Factors

Characteristics of the adult rat population and environmental factors in relation to rat fertility are summarized in table 1. Mean maternal weight at mating was 276±21.8g (220-375g) at a mean age of 14±2.9 weeks (11-29 weeks). There was a strong correlation between age and weight (p<0.001). Mean paternal age at mating was 28±3.6 weeks (13-37 weeks). Mating was performed on every day of the week.

Pregnancy Rate, its Prediction and Litter Size

Pregnancy rate was 74% after the first mating attempt. 11, respectively, 3 female rats underwent a second or third mating attempt resulting in a subsequent pregnancy in 64% and 67% (n.s., table 1). Five percent of female rats developed pseudopregnancies after repeated vaginal smears, which resulted in a significantly lower pregnancy rate thereafter (p<0.05, table 1) Neither the specific day, the year nor FBZ exposure had a significant effect on pregnancy rate. Clidox-S application was only significant in the univariate (p<0.05) not the multivariate analysis (table 1).

We compared three methods to predict successful mating: (1) the presence of sperm in the vaginal smear, (2) the presence of a vaginal plug after mating and (3) a maternal weight gain ≥15% at ED9 of a potential pregnancy. All 3 parameters were strongly correlated with the occurrence of an actual pregnancy (p<0.001). The presence of only sperm fragments rather than complete sperm or a mixture of any amount was most indicative of a subsequent pregnancy (98.4% versus 87%, p<0.001). Positive and negative predictive values of these methods are listed in table 2.

Of 227 pregnant dams 200 had received NF and 27 did not. Mean litter size was 13 in NF+ (range 1-20) as well as NF- (range 3-16) groups. Litter size was smaller in females with previous pseudopregnancies (mean litter size of 7 versus 13; p=0.0025). FBZ and Clidox-S exposure did not have an impact on the total litter size.

Effects of Nitrofen upon Gestation

The mean rate of spontaneous abortions of any gestational age (GA) was 8.5% (0-83%) in NF+ fetuses compared to 3.9% (0-33%; n.s.) in NF- fetuses. Of the 1961 NF+ fetuses, 37% had LCDH, 7% RCDH and 3% BCDH. NF+ pups without CDH had a lower lung and body weight compared to NF- pups (p<0.001). The fLBWR, a measure of pulmonary hypoplasia, was lower in NF+ fetuses with CDH than those without (p<0.05). The effect on fLBWR of the combination of CDH and NF-exposure was more pronounced later in pregnancy, suggesting the progressive nature of the disease. In the absence of CDH, NF-exposure is not associated with a decreased fLBWR near term (table 3).

Effects of Fetal Surgery on Fetal Survival

The survival rates after fetal surgery on ED18.5 were 96% in untouched, 76% in sham-operated and 66% in TO fetuses (TO/Sham vs. control p<0.001). Later surgery resulted in higher survival rates: Intervention at ED19 resulted in 97%, 87% and 83% surviving fetuses and at ED20 this was 94%, 88% and 92%, respectively (TO18.5 vs. TO19/20 p<0.01). The few animals which underwent interventions at ED18 had a higher mortality rate, so we abandoned this practice (unpublished experience of our group).

Discussion

We report in great detail outcomes of our large scale breeding and experimental surgery program for CDH in the NF-Wistar rat. With very consistent conditions in terms of personnel, housing and experimental circumstances, we document fetal lung development along 12h intervals near term. GA was well-defined by restricting mating to a 1h period over the entire experimental phase of three years.

Herein we obtained an overall pregnancy rate of 74% at first attempt, a fetal loss rate (before interventions) of 8.5% for NF+ fetuses which did not significantly differ from NF- fetuses (3.9%) and an average litter size of 13. Humphreys (1976) observed at

Table 1: Pregnancy rate and influencing factors: Variables were tested in univariate and multivariate stepwise regression analysis. Arrows symbolize a positive (↑) or negative (↓) effect on pregnancy rate. The cut-off at which 60% of animals become pregnant is given if applicable and a p-value is specified. In case of nominal variables the best and worst setting is reported. FBZ exposure was recorded at any time before or during pregnancy. Clidox-S exposure was recorded at the mating day or during pregnancy.

Independent variable	Type of effect	60% cut-off	Univariate analysis	Multivariate analysis (R^2=0.13)
Female weight (grams)	↓	300	<0.0001	0.0249
Female age (weeks)	↓	18.5	0.0009	n.s.
Male age (weeks)	↓	35	0.0006	n.s.
Number of mating attempt			n.s.	
Previous pseudopregnancy	↓		0.0004	0.0147
FBZ exposure			n.s.	
Clidox-S exposure	↓		0.0347	n.s.
Day of the week/ Year			n.s.	

FBZ= fenbendazole, n.s. = not significant.

Table 2: Predictive values of different detection methods for pregnancy at ED9: The presence of a vaginal plug was determined at latest seven hours post mating. Vaginal smears to check for the presence of sperm were taken directly after mating. The weight gain (%) was determined between the time-point of mating and the application of NF on ED9 of a potential pregnancy. Data was available on 297 mating attempts.

	Vaginal plug	Vaginal sperm	Weight gain ≥15%
PPV	92.7%	91%	94.4%
NPV	78.5%	67.8%	45.5%

PPV= positive predictive value, NPV= negative predictive value.

maximum a 97.4% pregnancy rate when leaving virgin Sheffield-Wistar dams at 12 weeks age with males until detection of a plug or obvious pregnancy resulting in an average of 9.3 pups born per mated female. An alternative strain or age resulted in a lower pregnancy rate.[24] Similar results were published by Rutledge (1974).[25] Given these historical findings results to our limitations in mating conditions seem very acceptable.

In order to document lung development, we aimed to obtain normative data in healthy and NF+ fetuses (table 3). Each experimental subgroup contained at least 30 fetuses, on average 62.6 fetuses could be evaluated. We consistently observed values with little variation. This may allow for reduction of subjects in future experiments, in accordance with concerns of animal well-being.

In NF- pups, the lung grows faster than the body, peaking at ED19.5. In nitrofen-exposed CDH- fetuses this effect can as well be observed with a peak in fLBWR at ED20. In LCDH fetuses the fLBWR is highest at ED19 and decreases thereafter. The latter is known as the progressive nature of lung hypoplasia towards term and observed in animal studies as well as the clinical setting. To obtain severe disturbances in lung development, pups must not only be NF+ but also have CDH. At term LCDH pups have a fLBWR of 60% compared to that of NF-exposed littermates without CDH. Strictly speaking the fLBWR of LCDH fetuses of 1.7% at ED21 does not meet the pathologic criteria of pulmonary hypoplasia used clinically (fLBWR ≤1.5% until 27wks, ≤1.2% 28wks until term).[26] NF+ fetuses without CDH, have a fLBWR within the normal range. Their lungs weigh 30% less than in NF- pups, but the effect on fLBWR is offset by a coinciding decrease in fBW. In conclusion it seems mandatory for researchers studying the pathophysiology of pulmonary hypoplasia as well as the effects of fetal therapy to focus on findings in NF+ fetuses with CDH. The distinct course of lung growth in the described subsets needs to be addressed when comparing respective data.

The occurrence of CDH in our study was 47% in total, 37% of fetuses had left-sided lesions. Our total rate was comparable to the 42% of Kluth,[11] but considerably less than the 75% described by Brandsma.[16] We could not reproduce the majority of RCDH[11] or their total absence.[16] One obvious difference with both studies was the rat strain used: Sprague-Dawley embryos are known to be more susceptible to NF exposure (as summarized by Beurskens 2007[27]), although this explains the findings only in part. Kluth exposed their rats to NF by mixing it into food and leaving this for a 12h-intake period after 24h of fasting.[11] Brandsma used a gastric tube as we did.[16] Both studies do not reveal the exact hours of NF exposure which would also be relative given the overnight-mating protocol.

One of the main goals of our group is to reverse pulmonary hypoplasia prenatally. Fetal TO is one option[28,29] as we have already reported in detail.[20] Our experiments show that fetal surgery can be done at a reasonable price. At ED18.5 survival after TO was 66%, increasing up to 92% for interventions performed at ED20. This allows for a reasonable number of animals in the experiments as it reflects our overall numbers not even considering the team's learning curve.

Table 3: Fetal biometrical data in the different groups: Data are presented as mean ± standard error of the mean. NF- fetuses represent healthy controls. NF+ animals are subdivided depending if they had a left-sided (LCDH) or no defect (CDH-). The number of fetuses is given in brackets. In the statistical comparison of healthy fetuses (NF-) of different gestational age (GA), columns marked with the same symbol depicted no significant difference. In the statistical analyses of NF+ fetuses the significance level is indicated.

	ED19			ED19.5			ED20			ED20.5			ED21		
	NF- (n=55)	CDH- (n=43)	LCDH (n=31)	NF- (n=39)	CDH- (n=61)	LCDH (n=39)	NF- (n=52)	CDH- (n=70)	LCDH (n=40)	NF- (n=39)	CDH- (n=81)	LCDH (n=57)	NF- (n=43)	CDH- (n=173)	LCDH (n=116)
fBW (mg)	2,496±27	1,987±25	2,034±31	2,549±57	2,236±34	2,187±44	4,144±57	2,934±36	2,957±60	4,392±68	3,275±44	3,311±50	5,926±55	4,197±33	4,081±90
fTLW (mg)	82±1.6	54±1.1	50±1.4	89±2.6	62±1.4	54±1.3	136±2.1	91±1.4	70±1.9	137±3	96±2	74±1.4	168±3	119±2.6	71±2.6
fLLW (mg)	28±0.5	19±0.5	17±0.5	30±0.9	22±0.5	18±0.5	45±0.8	32±0.6	23±0.6	46±1.1	34±0.7	23±0.5	56±1.1	41±0.9	22±0.9
fLBWR (%)	3.3±0.05	2.7±0.05	2.5±0.06	3.5±0.05	2.8±0.05	2.5±0.05	3.3±0.04	3.1±0.04	2.4±0.06	3.1±0.04	2.9±0.04	2.3±0.05	2.8±0.06	2.8±0.05	1.7±0.05
NF- vs. NF- at different GA or NF+ at same GA:															
fBW	#	***	***	#	***	***	+	***	***	+	***	***		***	***
fTLW	#	***	***	#	***	***	+	***	***	+	***	***		***	***
fLLW	#	***	***	#	***	***	$ +	***	***	$	***	***		***	***
fLBWR	$ #	***	***	# +	***	***		**	***		**	***		n.s.	***
LCDH versus CDH-:															
fBW		n.s.			n.s.			n.s.			n.s.			n.s.	
fTLW		n.s.			**			***			***			***	
fLLW		*			***			***			***			***	
fLBWR		*			***			***			***			***	

n.s. = not significant; * = p<0.05; ** = p<0.01; *** = p<0.001. ED= embryonic day, fBW= fetal body weight, fTLW= fetal total lung weight, fLLW= fetal left lung weight, fLBWR= fetal lung-body-weight ratio.

In laboratory animal research based on breeding colonies it is difficult to standardize certain conditions. For instance the age of animals in the colony will increase and as a consequence their weight which negatively affects fertility. An alternative would be to order time-mated dams, however, this reduces control over other experimental factors. A strategy to limit the potential extent of such unwanted effects should be to perform experiments in the shortest time frame possible.

Conclusion

We provide data that may help to calculate numbers needed in a breeding colony for experiments on fetal lung development. Limiting the mating interval, which is a requirement for this type of research, still provides reasonably high pregnancy rates and a large litter size. Exposure to NF as well as fetal surgery is associated with high fetal survival rates. In addition, lung findings were very reproducible and eventually will reduce the number of animals within experimental subgroups.

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Conflict of interest

None of the authors has a conflict of interest.

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