

FINAL VERSION

**REDUCTIONS IN THE INCIDENCE OF NITROFEN-INDUCED DIAPHRAGMATIC
HERNIA BY VITAMIN A AND RETINOIC ACID**

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ABSTRACT

Congenital diaphragmatic hernia (CDH) is a serious medical condition in which the developing diaphragm forms incompletely, leaving a hole through which the abdominal contents can enter the thoracic space and interfere with lung growth. A perturbation of the retinoid system has been linked to the etiology of CDH. This includes findings that nitrofen, which induces CDH in rodents, inhibits the key enzyme for retinoic acid production, retinaldehyde dehydrogenase-2 (RALDH2) *in vitro*. Published studies indicate that antenatal vitamin A administration on gestational day (D) 12 in the nitrofen model of CDH reduced the severity and incidence of right-sided defects and lung hypoplasia. In this study, we administered nitrofen on D8, to include the induction of clinically more prevalent left-sided defects, and examined the efficacy of several vitamin A administration paradigms to gain insights into the developmental stage of susceptibility. Further, we tested the hypothesis that administration of retinoic acid, the product of RALDH2 activity, is more potent than administering the substrate, vitamin A, in reducing the incidence of CDH. The incidence of CDH was reduced from ~ 54% (nitrofen alone) to ~32% with vitamin A treatment. The efficacy of RA treatment was very marked with a reduction in the incidence of CDH to ~15%. Administration of vitamin A or RA on ~D10 was most effective. These data lend further support for the potential involvement of retinoid signaling pathways and the etiology of CDH and supports data from *in vitro* studies demonstrating a nitrofen-induced suppression of RALDH2.

Key words: diaphragm, respiration, newborn, lung

INTRODUCTION

Congenital diaphragmatic hernia (CDH) occurs in ~1:3000 live births (11). It is characterized by failure of a portion of the diaphragm muscle to form during development. This hole in the diaphragm can lead to invasion by the developing viscera of the abdominal compartment into the thoracic space, thus interfering with lung growth and development (14). Infants born with CDH suffer from severe respiratory failure due to lung hypoplasia and pulmonary hypertension being the most serious. The cost of treatment of CDH and its attendant complications is high and, despite technological advances in equipment and surgical techniques, the mortality and morbidity remains severe (21). As a result, considerable research into the treatment and prevention of CDH and its associated problems is in progress. CDH research has benefited from the existence of an animal model of CDH. The well-established nitrofen model (8) has provided a means of producing a condition in rodents very similar to that observed in human CDH (18).

Data from the literature have suggested that the retinoid system may be involved in CDH (reviewed in 10). In 1941, Andersen (3) published data that showed that there was a 25% incidence of CDH in the litters of vitamin A-deficient rats. Diaphragmatic hernias were documented in ~15% of the offspring of double retinoic acid receptor knockout mice (16). A small clinical study showed that mothers and their infants with CDH had lower plasma retinol levels compared with the levels in mothers and healthy infants (15). A study using genetically engineered mice demonstrated a pronounced suppression of the retinoid response element by nitrofen (6). Further, it has been demonstrated, using an *in vitro* assay, that nitrofen inhibits mammalian retinaldehyde dehydrogenase-2 (RALDH2), the enzyme catalyzing the final step in

retinoic acid production (17). Collectively, these data strongly imply that the retinoid system and/or factors influenced by retinoids are candidates for involvement in the etiology of CDH.

Administration of large doses of vitamin A antenatally reduced the incidence of nitrofen-induced CDH in rats from ~80% to ~50% (22). In that study, nitrofen and vitamin A were administered at gestational age (D)12, which produces solely right-sided defects. In this study, we examine the ability of several regimes of vitamin A administration to counter the effects of nitrofen administration on D8. This allowed us to test the efficacy of vitamin A in a paradigm that induced left-sided defects (more typically observed clinically) and to gain some insights into the developmental stage of susceptibility. Further, we tested the hypothesis that administration of retinoic acid, the biologically active form of vitamin A, will be much more potent than vitamin A in reducing nitrofen-induced CDH. The rationale for the hypothesis is based on the fact that nitrofen inhibits RALDH2 *in vitro*. If this translates to the *in vivo* state, then one would predict a differential efficacy of vitamin A versus retinoic acid administration.

MATERIALS AND METHODS

Nitrofen Model of CDH. Timed-pregnant Sprague-Dawley rats (morning of detection of sperm-plug was assigned D0) were provided by the Health Sciences Laboratory Animal Services of the University of Alberta. Nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether; 100 mg) was dissolved in olive oil (1ml) by gentle sonication. Pregnant rats were anaesthetized (~10 min) using halothane (1.25% in 95% O₂-5% CO₂) and nitrofen was administered via gavage tube in the afternoon on D8. All protocols were approved by the Animal Welfare Committee at the University of Alberta

Vitamin A Administration. Vitamin A (25,000 IU; Avibon, Rhone-Poulenc, Paris, France) was mixed with 0.5 ml olive oil and administered via gavage tube to anaesthetized pregnant rats. Animals were assigned to 4 groups: one group received a single dose of vitamin A on the same day as nitrofen (D8), the second was given nitrofen on D8 and vitamin A on D10, a third group received vitamin A and nitrofen on D8 and vitamin A on D10 and a fourth was given vitamin A and nitrofen on D8 with doses of vitamin A on D10 and D12. A fifth group was fed the same weight of food treated with olive oil alone to serve as controls. Fetal rats (D19-21) were delivered by caesarean section, euthanised, fixed in 4% paraformaldehyde, and dissected under a Leica dissecting microscope to determine the presence of diaphragmatic hernias.

Retinoic Acid Administration. Initial measures of food intake determined that the dams were eating approximately 22 g of rodent chow/day. Thus, 22 g of standard rodent chow was immersed in warm water for 3 minutes in ceramic feeding bowls to soften the pellets and increase their absorbency. *All-trans* retinoic acid (Sigma Chemical Company, USA) was suspended in olive oil, added (1.5 mg *all-trans* retinoic acid per 4 g of food pellets), and allowed to infiltrate the pellets (variation of methodology described in ref. 24). The bowls containing the treated food were placed in the cages and were the only source of food. The nitrofen-treated animals were fed 22 g of food with added *all-trans* retinoic acid daily from D8 to D13 in the first group, D8 to D10 in a second group, or on D8 alone in the final group. Some animals were fed the same weight of food treated with olive oil alone to serve as controls. Fetuses were delivered by caesarean section on D16, euthanised, fixed in 4% paraformaldehyde, and dissected under a Leica dissecting microscope to determine the presence of diaphragmatic hernia.

Statistic Analysis. Data are expressed as means +/- SD. Inter-group differences in the retinol and retinoic acid protocols were determined by unpaired t tests using the program GraphPad Prism (GraphPad Software, Inc.) P values less than 0.05 were considered significant.

RESULTS

Incidence of CDH in response to nitrofen delivery: The incidence of diaphragmatic defects in response to nitrofen administration was 54% (N=237). It is critical when assessing the efficacy of vitamin A and retinoic acid suppression of CDH to have an accurate measure of CDH incidence. Examination of the literature concerning nitrofen and the production of CDH shows that nitrofen induces hernias in rat fetuses from pregnant animals treated on D8 or D9 at a rate of between 41-69% (mean=54.5; n=12 studies; Table 1).

Incidence of CDH with vitamin A treatment: The incidence and types of hernias in response to vitamin A treatment are shown in Table. 2. Treatment with vitamin A on D8 in conjunction with nitrofen did not significantly reduce the incidence of CDH. Vitamin A administered on D8 and 10 or 8, 10 and 12 or D10 only, reduced the incidence of CDH significantly. Nitrofen or nitrofen-vitamin A treatment did not adversely affect fetal survival.

Incidence of CDH with retinoic acid treatment: The incidence of types of hernias in response to all-*trans* retinoic acid treatment is detailed in Table 3. There was a profound reduction in the incidence of nitrofen-induced hernias in conjunction with all of the retinoic acid paradigms used. No adverse effects were observed with supplementation of the food with 1.5 mg of all-*trans* retinoic acid daily. The numbers of fetuses per animal was the same as seen in

untreated animals and no gross external malformations such as those indicative of retinoic acid teratogenesis were observed in the fetuses examined.

Table 1 summarizes the incidence of diaphragmatic hernias and statistical differences between nitrofen, nitrofen + vitamin A and nitrofen + retinoic acid groups.

DISCUSSION

In this study, we administered nitrofen on D8 to generate left-sided, right-sided and bilateral diaphragmatic hernias (2). Co-administration of large doses of vitamin A in conjunction with nitrofen on D8 did not reduce the incidence of hernias. However, antenatal treatment with vitamin A on D8 and D10, D8, D10 and D12 or D10 did result in a statistically significant decrease in nitrofen-induced CDH (~33% incidence compared to 54% without vitamin A). This observation is consistent with the study by Yu et al. (27) who reported a trend for the reduction in the incidence of hernias induced by D9 nitrofen administration in response to administration of vitamin A on D10. The authors propose that the data did not reach statistical significance due to the small number of experimental animals used for that study. Our focus during this study was on diaphragmatic defects. However, the earlier studies by Thébaud et al (22, 23) and Yu et al. (27), clearly demonstrate the vitamin A administration can reduce the teratogenic effects of nitrofen on the lung, thymus and heart.

The administration of all-*trans* retinoic acid was markedly more effective in reducing nitrofen-induced hernias compared to those observed with vitamin A administration. Provision of RA-treated food on D8, concomitant with nitrofen exposure, decreased the incidence of hernias to ~ 15% compared to 54% with nitrofen treatment alone. If the nitrofen-exposed animals were

provided with RA-supplemented food for 3 or 5 days following nitrofen administration, a further decrease in hernia incidence to less than 10% was observed.

An *in vitro* study has previously demonstrated that nitrofen suppresses the action of RALDH2, the key enzyme necessary for conversion of retinal to retinoic acid. This would suggest that the decreased enzyme activity could be partially countered by increasing the amount of substrate. That is consistent with the partial reduction in hernias by the administration of very large doses of vitamin A used in this and past studies (22, 27). In contrast, administration of all-*trans* retinoic acid bypasses the enzymatic perturbation and thus could explain the marked increase in the efficacy of hernia reduction by all-*trans* retinoic acid versus vitamin A.

Based on the efficacy of hernia reduction of the various treatment paradigms for both vitamin A and retinoic acid, it would appear that the time-period around D10 is of particular importance. Data from past studies (2, 4, 9) suggests that a defect in the mesenchymal tissue within the primordial diaphragm, the pleuroperitoneal fold (PPF), is underlying the pathogenesis of the diaphragmatic defect associated with nitrofen-induced CDH. It is known that RALDH2 and retinol binding proteins are strongly expressed in the PPF at E13 (5, 17), when diaphragm muscle precursors and phrenic axons have reached the PPF. Given the data from the current study, the focus now is to further understand the embryogenesis of the PPF at earlier ages and the role of retinoids in its development.

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Table 1: CDH Incidence in Response to Nitrofen in Sprague Dawley (SD) and Wistar Rats

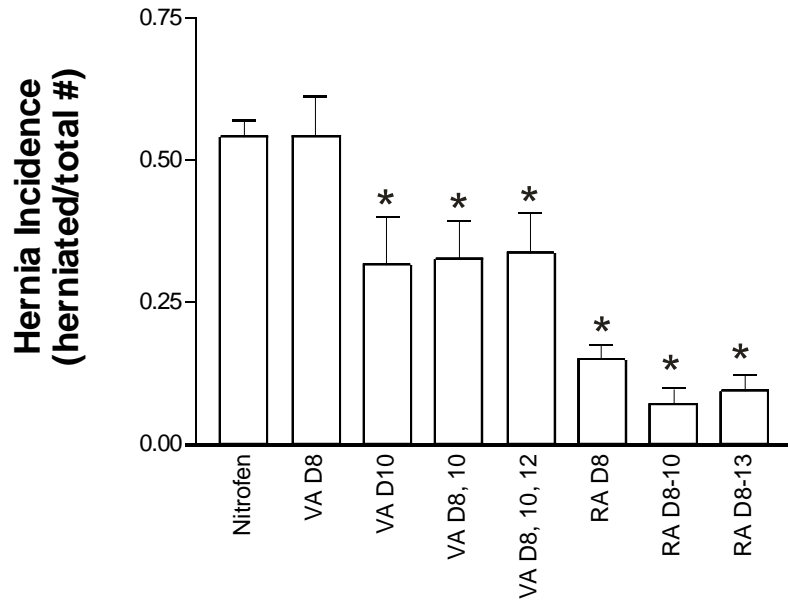
Day of nitrofen administration	Amount of nitrofen	Incidence of CDH	Reference
D8	100mg (SD)	54%	Current study
D9	100mg (SD)	52%	2
D9	100mg (SD)	56%	13
D9	100mg (SD)	61%	18
D9	100mg (SD)	48%	12
D9	100mg (SD)	63%	25
D9	100mg (SD)	53%	19
D9	100mg (Wistar)	46%	1
D9	100mg (SD)	69%	26
D9	100mg (SD)	41%	27
D9	100mg (SD)	68%	28
D9	100mg (Wistar)	44%	7
D12	75mg (SD)	60%	20
D12	100mg (SD)	84%	22

Table 2: CDH Incidence Under the Vitamin A Protocol

Treatment	Left-sided hernias	Right-sided hernias	Bilateral hernias	Total hernias	Number of fetuses	% hernias observed
Vehicle	0	0	0	0	41 (n=3 litters)	0
Nitrofen D8	133	80	24	237	437 (n=31 litters)	54%
Retinol D8	13	31	8	51	93 (n=7 litters)	55%
Retinol D8, D10	21	8	1	30	91 (n=6 litters)	33%
Retinol D8, D10, D12	9	14	1	24	74 (n=5 litters)	32%
Retinol D10	8	14	1	24	75 (n=5 litters)	32%

Table 3: CDH Incidence Under Retinoic Acid Protocol

Treatment	Left-sided hernias	Right-sided hernias	Bilateral hernias	Total hernias	Number of fetuses	% hernias observed
Vehicle	0	0	0	0	54 (n=4 litters)	0
Nitrofen D8	133	80	24	237	437 (n=31 litters)	54%
RA D8	3	8	0	11	73 (n=5 litters)	15%
RA D8-10	2	5	0	7	77 (n=5 litters)	9%
RA D8-D13	7	5	0	12	148 (n=10 litters)	8%



Graph 1: Overall incidence of diaphragmatic hernias in response to nitrofen alone and in conjunction with vitamin A (VA) or all-*trans* retinoic acid (RA). The incidence of herniation was significantly (* - $p < 0.05$) lower with vitamin A or retinoic acid administration compared with nitrofen alone.