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Lack of Sex Differences in Antioxidant Enzyme Development in the Fetal Rabbit Lung

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ABSTRACT. A sex difference characterized by a female advantage in the maturation of the fetal pulmonary surfactant system is well documented. Because the surfactant system and the antioxidant enzyme system of the fetal lung have chronologically similar developmental patterns and share some of the same hormonal regulators, such as glucocorticoids, we questioned whether a sex difference would be present in antioxidant enzyme maturation as it is in surfactant system maturation. We studied fetal rabbits at days 26 and 28 of a 31-day gestational period. Fetal sex was identified histologically. Fetal lung lavage was performed and lavage fluid assayed for phosphatidylcholine, disaturated phosphatidylcholine, and sphingomyelin. Lung tissue from separate fetuses was assayed for disaturated phosphatidylcholine content and total phospholipid content and for the activities of three antioxidant enzymes—superoxide dismutase, catalase, and glutathione peroxidase. No differences were present in antioxidant enzyme maturation between male and female fetal rabbits at the gestational days studied. A female advantage was observed in the lung lavage disaturated phosphatidylcholine/sphingomyelin ratio (at 26 days: female 1.38 ± 0.42, male 0.99 ± 0.26; and at 28 days: female 3.29 ± 0.53; male 2.26 ± 0.35, $p < 0.05$). A female advantage in surfactant development was not reflected in lung tissue disaturated phosphatidylcholine or total phospholipid. We conclude that, unlike the development of the surfactant system, the development of the antioxidant enzyme system in the fetal rabbit lung does not demonstrate a sex difference. (*Pediatr Res* 26: 16–19, 1989)

Abbreviations

DSPC, disaturated phosphatidylcholine
TPL, total phospholipid
AOE, antioxidant enzymes
SOD, superoxide dismutase
CAT, catalase
GP, glutathione peroxidase
PC, phosphatidylcholine
S, sphingomyelin
HMD, hyaline membrane disease
BPD, bronchopulmonary dysplasia

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The surfactant system and the antioxidant enzyme system of the fetal lung share chronologically similar developmental patterns. Both systems demonstrate marked increases during the final 10–15% of gestation (1), and both are important in assuring the successful functioning of the neonatal lung in the relatively oxygen-rich *ex utero* environment. While the surfactant reduces alveolar surface tension to prevent alveolar collapse at end-expiration, the AOE:SOD, CAT, and GP function to detoxify the highly reactive O_2 metabolites produced intracellularly during normal aerobic metabolism and at greater rates under hyperoxidant conditions (2, 3).

In addition to parallel developmental patterns, the surfactant system and antioxidant enzyme system exhibit certain similarities in hormonal regulation. Exogenous glucocorticoid administration to pregnant rats in late gestation produces similar accelerations in the normal maturation of fetal lung DSPC content (the major phospholipid component of surfactant) and AOE activity (4). Conversely, late gestational blockade of endogenous glucocorticoid production with metyrapone results in delays in both fetal rat lung surfactant and AOE development (5). The late gestational maturation of the fetal lung is advanced in the female fetus of several species, including the human, rabbit, rat, and mouse (6). Examples of this earlier maturation in the female include the advanced development of mature type II cells and remodeling of the alveolar structure (7), the synthesis of surfactant (8, 9), and earlier β-adrenergic innervation and receptor development (10). In addition, the response of the fetal lung to the glucocorticoid regulation of surfactant maturation also exhibits this sex difference (6). The parallels noted in development and hormonal regulation of the surfactant system and the AOE suggested that a similar female advantage might be found in the developmental ontogeny of the AOE system. We undertook our study to determine whether the female advantage associated with surfactant maturation would characterize AOE maturation as well.

MATERIALS AND METHODS

Pregnant New Zealand White rabbits were obtained from K-W Farms, Tice, FL. The exact breeding times (±6 h) were provided by the supplier. Pregnant rabbits were anesthetized with intravenous pentobarbital; fetuses were delivered by hysterotomy and killed with pentobarbital before air breathing at days 26 and 28 of a 31-day gestation period. Fetal gonads were identified grossly by shape, color, and position. Gonads were then removed, placed in buffered formalin, sectioned and stained with hematoxylin and eosin. The sex of each fetus was definitively determined by microscopic examination.

Random litters were selected for assessment of lung tissue DSPC and total phospholipid content and for antioxidant enzyme activities. After fetal death, fetal lungs were perfused immediately *in situ* via the pulmonary artery using cold saline. The

perfused lungs were removed, stripped of nonpulmonary tissue, weighed and homogenized in 20–30 times their wt of cold saline in a Brinkmann polytron (high speed, 90 s).

Aliquots of the lung homogenate were subsequently analyzed for AOE activities using standard spectrophotometric assays for SOD (11), CAT (12), GP (13), and DNA and protein (14, 15). All assays were done on coded samples without knowledge of the fetal sex. Purified enzyme standards (SOD and CAT) and DNA standard were obtained from Sigma Chemical Co. (St. Louis, MO) and Boehringer Mannheim (Indianapolis, IN) (GP). Results of antioxidant enzyme analyses were expressed as units of enzyme activity per mg DNA (and also calculated per mg protein and per g wet lung wt).

Lipid extraction of the lung homogenate aliquot was performed according to the method of Bligh and Dyer (16). The lipid extract, once it was dried under nitrogen, was frozen before phospholipid analysis. An aliquot of lipid extract was set aside for measurement of TPL. A second aliquot was assayed for DSPC using the procedure of Mason *et al.* (17). After the DSPC was separated from the other phospholipids, the DSPC and TPL samples were assayed for inorganic phosphorus using the method of Morrison (18). A known quantity of [^{14}C]dipalmitoyl phosphatidylcholine (New England Nuclear, Boston, MA) was added before lipid extraction and aliquots counted at each step to correct for sequential losses (representing <10% for TPL, and <20% for DSPC). Lipids were expressed as mg/g wet lung wt (and also calculated per mg protein, and as a ratio of mg DSPC to mg TPL).

In litters randomly selected for lung lavage, fetuses underwent tracheal cannulation with a 16-gauge blunt adapter after death. Lavage was performed with 0.9% ice-cold saline in aliquots of 0.5 ml and repeated five times. A total of 80–90% of the instilled saline was recovered from both sex groups of fetal animals. Lavage samples were coded to prevent knowledge of the fetal sex and subsequently were assayed for content of DSPC, PC, and S in a separate laboratory by one of the investigators (HCN) who was unaware of the fetal sex. Samples that were contaminated with blood were not assayed. (These eliminated samples were not significantly different in sex distribution and represented ~15% of the collected samples.) Fluids were extracted by the method of Folch *et al.* (19), and split into two equal aliquots. PC and S were isolated from one aliquot by thin-layer chromatography in chloroform:methanol:water 65:35:5. The other aliquot was treated with osmium tetroxide and DSPC was isolated by thin layer chromatography in the same solvent (8). PC, DSPC, and S were then quantitated by phosphorus assay (20). The results were summarized as the ratios PC/S and DSPC/S. Samples in which the S measurement was at the lower limit of assay (representing approximately 20% of the collected samples, equal numbers of male *versus* female) were not included in the final analysis. At this point, the code was broken and results for male and female lavage PC/S and DSPC/S were compiled. There were no differences between male or female groups in the number of samples which were omitted because of blood contamination or extremely low S values.

Statistics were performed using Student's *t* test (21). Two-tailed comparisons were used for evaluation of AOE. One-tail comparison (female > male) were used for lung lavage and tissue phospholipid comparisons. The abundance of evidence supporting the female advantage in fetal rabbit lung lavage made this decision reasonable.

RESULTS

Composite body and lung wt of fetuses from 26–28 days of gestation demonstrated no differences between male and female fetuses when either lung or body wt measurements or lung/body wt ratios were compared (*e.g.* mean lung/body wt at 26 days = 2.62 and 2.84%; at 28 days = 2.88 and 2.85%, male *versus* female, respectively).

The gestational progression of lung DSPC and total phospholipid content in male and female fetal lungs at 26–28 days of gestation is seen in Table 1; no apparent differences in either lung DSPC or TPL content were present between male and female rabbit fetuses of gestational age 26 or 28 days whether expressed per g of lung or per mg of protein (data not shown).

The results for lung lavage PC/S and DSPC/S ratios are given in Figure 1. No differences between males and females at either gestational age were found for the PC/S ratio. The DSPC/S ratio showed a trend at 26 days for higher values in the female (mean increase = 39%) and at 28 days, female fetuses demonstrated definitely elevated DSPC/S ratios ($p < 0.05$) compared to males (mean increase = 46%).

Figure 2 illustrates the activities of the three pulmonary antioxidant enzymes, SOD, CAT, and GP, for male and female fetuses at gestational days 26 and 28. No significant differences

Table 1. *Lung DSPC and total phospholipid content in male and female fetal rabbits (mean ± SE)**

	DSPC	TPL	DSPC/TPL
26 Days of gestation			
Male (n = 18 fetuses)	1.41 ± 0.05	9.20 ± 0.20	0.153
Female (n = 14 fetuses)	1.42 ± 0.04	9.42 ± 0.20	0.151
28 Days of gestation			
Male (n = 16 fetuses)	1.90 ± 0.09	11.27 ± 0.35	0.168
Female (n = 24 fetuses)	2.01 ± 0.08	11.89 ± 0.28	0.169

* Units: mg/g lung wet wt. No differences are statistically different.

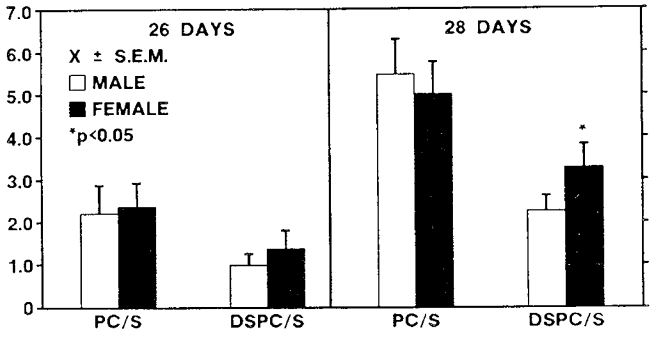

Fig. 1. Lung lavage PC/S and DSPC/S ratios in male and female fetal rabbits at 26 and 28 days of gestation. Values are mean ± SEM. * $p < 0.05$. n at 26 days: PC/S, male = 9, female = 14 fetuses; DSPC/S, male = 8, female = 13 fetuses. n at 28 days: PC/S, male = 15, female = 19 fetuses; DSPC/S, male = 20, female = 15 fetuses.

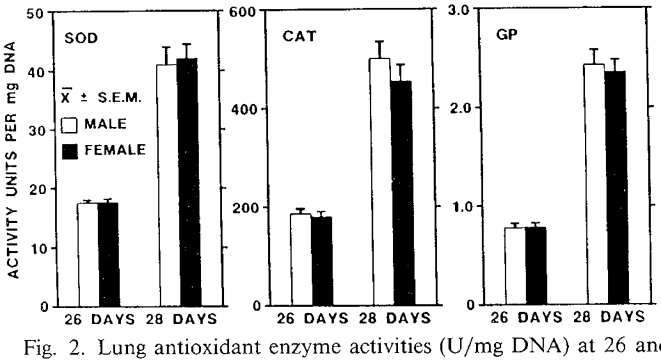

Fig. 2. Lung antioxidant enzyme activities (U/mg DNA) at 26 and 28 days of gestation in male and female fetal rabbits. Values represent means ± SE; n = 4 litters/gestational age. No differences between males and females are statistically significant.

were present between male and female fetuses in SOD, CAT, or GP activities at the two gestational days examined whether expressed per mg of DNA (Fig. 2) or per mg of protein or per g of lung (data not shown; largest mean difference ~10%).

DISCUSSION

The interest in the role that fetal sex might play in influencing lung maturation is based first on the recurrent clinical observation that male infants are at increased risk for developing HMD compared to female infants of equivalent gestational age (22); and second on the finding that glucocorticoid therapy of the fetus to stimulate surfactant synthesis appears ineffective in the male fetus (6). In investigating the explanation for these clinical observations, most studies have focused on the development of fetal lung surfactant production, surfactant-related lung function, and surfactant-related morphologic maturation in male and female fetal lungs from a variety of species. The "female advantage" in lung maturation in the rat was found by Adamson and King (7) to consist of increased lung DSPC content in 19- and 20-day gestation females as well as increased numbers of lamellar bodies in epithelial cells of female rats at 19–21 days of gestation. In the rabbit, multiple investigators have reported increased DSPC/S ratios in female rabbit lung lavage compared to males and more mature pressure/volume characteristics in female rabbit fetuses (8, 23, 24). The female fetal rabbit lung exhibits increased surfactant synthesis in response to glucocorticoids whereas the male does not (6).

The mechanism responsible for this female advantage in lung surfactant maturation likely relates to sex hormones. While estrogen has been demonstrated to stimulate fetal rabbit surfactant production (25), most work to date indicates that androgen exerts an inhibitory influence on fetal lung surfactant production (6, 26). Of interest, but adding some confusion to the situation, is the pattern of surfactant maturation that characterizes the developing monkey. Female fetal monkeys appear to have a "disadvantage" in surfactant maturation (27).

The maturation of both the surfactant system and the AOE system is crucial in preparing the fetal lung for its postnatal functioning and exposure to a relatively O_2-rich environment. It has now been carefully documented in at least four species—rat, rabbit, guinea pig, and hamster—that the late gestational increase in pulmonary AOE levels parallels the chronological development of the surfactant system during the final 10–15% of gestation, with maturational patterns that are remarkably similar between the two biochemical systems (1). In addition to their chronologically similar developmental patterns, both the surfactant system and AOE system appear to share some of the same hormonal influences. Specifically, dexamethasone administration to pregnant rats resulted in accelerated development of both the surfactant and antioxidant enzyme systems; conversely, inhibition of endogenous glucocorticoids by metyrapone administration produced comparable delays in development of pulmonary surfactant and antioxidant enzymes (4, 5). Because of the similar developmental patterns, the similar influence of glucocorticoid hormones on the surfactant and AOE systems, and because of the female advantages in surfactant maturation, we hypothesized that there would be a female advantage in the rabbit in antioxidant enzyme maturation as well.

The results of our study demonstrated neither a female nor a male advantage when AOE activity levels were examined. We did not explore whether male/female differences would be present if synthesis rates of the AOE were analyzed rather than activity levels, but given the equivalent activity results, both synthesis and degradation rates would have to be elevated in one or the other sexes to give the results in Figure 2. In addition, we did not investigate the possibility that a male/female difference would be present in AOE maturation specific to alveolar epithelial cells or any other single specific cell type. [The AOE are intracellular enzymes present in all lung cells (28).] Another possible explanation as to why a male/female difference was not demonstrated in AOE maturation in our study includes the possibility that male/female differences are present, but extremely short-lived, and occurring on a single day of gestation not explored by us (*i.e.* 25, 27, or 29 days). Such a result seems unlikely, as previous data show the sex difference in fetal rabbit surfactant production is manifested over at least 3 days (8). Inasmuch as sex differences in fetal surfactant maturation may be species specific, it is possible that in species other than the rabbit, sex differences in AOE maturation might be present. Finally, we did not separate the activities of CuZn SOD from Mn SOD. It is theoretically possible that a sex difference could be present in the development of Mn SOD (found in much smaller amounts in the lung than CuZn SOD, approximately 1:3–5) which could have been masked by our measurement of total SOD activity.

It is not surprising that we found no sex differences in lung tissue DSPC content or total phospolipid content. This has been found previously by one of us (Neilsen HC, and Torday JS, unpublished data) and reported by others (9, 30). Because lung tissue DSPC represents additional nonsurfactant related sources of DSPC as well as the DSPC of surfactant, it is likely that small but significant surfactant-related DSPC differences are obscured. We did document a sex difference in the DSPC/S ratio in the lung lavage. Although the magnitude of the difference was somewhat weaker than what has been previously reported (8), we believe that we have sufficient evidence to warrant a conclusion that a sex difference is present in the development of the surfactant, but not in the development of the AOE system in the fetal rabbit.

In the human infant, despite the epidemiologic evidence of increased HMD incidence in male infants, there are no convincing data to suggest that male infants (with the same degree of HMD, oxygen/ventilator exposure, gestational age, etc) are more at risk for developing BPD than female infants. Although the etiologic factors in the development of BPD are numerous and quite complex, O_2 toxicity is almost certainly a key factor, and it is likely that AOE immaturity in premature infants plays an important role (31). Although we examined the ontogeny of the antioxidant enzymes in the male and female fetal rabbit and did not study comparative susceptibility to O_2 toxicity, per se, our findings of a lack of sex difference in AOE development in the fetal rabbit would seem to be consistent with the lack of epidemiologic evidence of sex difference in the development of BPD in the human premature infant.

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Announcements

Pediatric Board Review

The Office of Continuing Education of the University of Michigan Medical School is sponsoring a Pediatric Board Review, September 11–16, 1989, at Towsley Center, Ann Arbor, MI.

This review will prepare participants for the pediatric board examination, as well as bring them up to date on pediatrics and its subspecialties. It is recommended for all senior residents, physicians interested in recertification, and those who had difficulty passing the examination.

For further information contact Betty Phillips, Program Assistant, Office of Continuing Education, G-1100 Towsley Center, Box 0201, University of Michigan Medical School, Ann Arbor, MI 48109-0201, (313) 763-1400.

National Conference on Pediatric Trauma

The Office of Continuing Education of the University of Michigan Medical School is sponsoring the National Conference on Pediatric Trauma, September 21–23, 1989, at Towsley Center, Ann Arbor, MI.

This conference is designed for pediatricians, pediatric surgeons, general surgeons, surgical specialists, emergency physicians, and nurses and will focus on progress and results in the management of life-threatening injuries to children, and the application of scientific methods to the study of pediatric trauma.

For further information contact Debra DeSmyther, Program Assistant, Office of Continuing Medical Education, G-1100 Towsley Center, Box 0201, University of Michigan Medical School, Ann Arbor, MI 48109-0201, (313) 763-1400.

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The Second Annual Carnation Symposium, "Nutrition During the Second Six Months of Life," is scheduled for July 12–14, 1989 at the Fairmont Hotel, San Francisco, CA.

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