Inositol and Glucocorticoid in the Development of Lung Stability in Male and Female Rabbit Fetuses[1]

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ABSTRACT. Inasmuch as inositol affects the development of lung surfactant, and exogenous glucocorticoids accelerate fetal lung maturation, a possible interaction of the two substances on alveolar stability of preterm rabbit fetuses of 28 days gestation was investigated. On days 26 and 27 of gestation inositol or glucose were added to the diet of does treated with betamethasone (0.2 mg/kg intramuscularly on days 26 and 27). Inositol increased lung-thorax compliance of paralyzed fetuses at all insufflation pressures studied (from 16 to 22.5 and 30 cm H_2O and back to 22.5, and 16 cm H_2O). At a ventilation pressure of 30 cm H_2O, lung-thorax compliance of fetuses treated with inositol plus betamethasone was more than doubled as compared with controls (1.2 ± 0.6 *versus* 0.5 ± 0.2 ml/kg × cm H_2O; $p < 0.001$). Inositol alone had no detectable effect on compliance, whereas betamethasone tended to increase compliance ($p = 0.05$). According to variance analysis, the effect of inositol was statistically significant only among the males. Inositol prevented the glucocorticoid-induced decrease in lung protein and, to a lesser extent, the decrease in DNA. Inositol did not further increase the lavageable surfactant pool of the glucocorticoid-treated, ventilated fetuses, although the area occupied by lamellar bodies within type II cells was increased after inositol plus betamethasone. According to the present study, inositol modifies the physiologic and biochemical response of the immature fetal lung to a pharmacologic dose of exogenous glucocorticoid. (*Pediatr Res* 24: 617–621, 1988)

Abbreviations

BM, betamethasone
BW, body weight
RDS, respiratory distress syndrome

The physiologic role of glucocorticoids in fetal lung maturation is widely acknowledged (1, 2). Exogenous glucocorticoids accel-

erate the maturation of the lung in various species (3–5) and prevent the RDS (6, 7). However, the magnitude of the effect on surfactant synthesis (8, 9) and secretion (10, 11) and particularly on the incidence of RDS (6, 11) is variable and not consistent with the notion that surfactant is the sole structure controlling lung maturity. Males exhibit a delay in fetal lung maturity and, according to some studies, a lack of responsiveness to glucocorticoid (6, 12). An additional poorly understood effect of exogenous glucocorticoids is the inhibition of lung growth (2, 5, 13).

In apparent contradiction to the proposed role of phosphatidylglycerol as an index of fetal lung maturity, addition of inositol supplement (known to suppress phosphatidylglycerol) (14–18) to the feeds of glucocorticoid-treated pregnant rabbits increased surfactant in alveolar lavage from fetuses (19). Furthermore, inositol added to the hormone-supplemented culture medium of fetal lung explants increased the synthesis of surfactant phosphatidylcholine (19).

Our investigation was undertaken in an attempt to clarify some of the controversies concerning the role of glucocorticoid in the development of the fetal lung. We evaluated whether exogenous glucocorticoid and/or inositol alters lung stability in the preterm fetus and whether the effect of these treatments depends on fetal sex.

MATERIALS AND METHODS

Animals. Experiments were performed on 31 healthy pregnant New Zealand White rabbits weighing between 3.5 and 4.5 kg, whose mating time was known to within ±3 h. Each rabbit was randomly assigned to the four treatment groups (group 1: seven does; group 2: seven; group 3: eight; and group 4: nine).

Protocol. On days 26 and 27 of gestation (day of mating = 0; term = 31) BM disodium phosphate (Bentelan, Glaxo) (0.2 mg/kg) was injected intramuscularly into the does of groups 3 and 4 and an equal volume of saline (0.25 ml/kg) into the animals of groups 1 and 2. Before BM and saline injections, 8% (wt/vol) inositol, 5 ml/kg, was infused intravenously during 5 min into the animals of groups 2 and 4, and glucose in the same fashion into the animals of groups 1 and 3. Starting from the time of the injection, glucose (groups 1 and 3) and inositol (groups 2 and 4) was added to drinking water at a concentration of 4% (wt/vol). Otherwise the animals received an ordinary laboratory food (Type C1, Spigadoro, Perugia, Italy) *ad libitum.*

Lung-thorax compliance measurements. On day 28 of gestation rabbits were anesthetized with 50 mg/kg of ketamine hydrochloride (Ketalar, Parke Davis), and the newborns were delivered under local anesthesia with 2% mepivacaine hydrochloride (Carbocaine, Pierrel). A fetus was injected intraperitoneally with 0.1 mg of pancuronium bromide (Pavulon, Organon), delivered by

hysterotomy, blotted and weighed. A tracheal cannula was se-
cured in the fetal trachea through a tracheostomy. Within 3–5
min from the delivery the fetus was attached to a body plethys-
mograph kept at 37° C, and a new fetus was delivered. The fetus
was ventilated with 100% O_2 at a frequency of 40 cycles/min
and an inspiration/expiration ratio of 5:6 with no end expiratory
pressure for a total period of about 5 min. Insufflation pressures
were changed every 60 s from "low" (16, 22.5 cm H_2O) to "high"
values (30 cm H_2O) and back to low again (22.5 and 16 cm
H_2O), in sequence. Tidal volume was continuously recorded by
means of a Fleisch tube connected to the pletysmograph, a
differential pressure transducer (DBF Engineering, Rome, Italy)
and a multichannel recorder (Physiograph, Houston, TX). The
respirator generated a square pressure wave and a plateau was
reached at end-inspiration. Lung-thorax compliance was calcu-
lated by dividing the tidal volume by the insufflation pressure,
and expressed on the basis of BW.

Biochemical analyses. After the period of artificial ventilation,
the fetuses were killed by intracerebral injection of sodium
pentobarbital. A total of 33 fetuses from the different treatment
groups was killed after hysterotomy without ventilation. The
lungs were lavaged *in situ* four times with saline (40 ml/kg); the
chest was opened and blood samples withdrawn by heart punc-
ture; the lungs were carefully excised, freed from contaminating
tissues, and blotted with filter paper. Blood samples and lungs
were stored at −20° C until analysis. Serum inositol of does and
their litters was measured as the trimethylsilyl derivative, using
gas liquid chromatography, as described previously (19). Lung
tissues were homogenized in ice-cold 0.05% Triton X-100 (wt/
vol, 1:4) with a tissue blender (Polytron, Kinematika, Switzer-
land) for 60 s, and aliquots taken for protein, DNA, and phos-
pholipid determinations.

Proteins were measured with the method of Bradford (20),
using bovine serum albumin as standard, and DNA by the
diphenylamine method of Burton (21) with fetal calf thymus
DNA as standard.

Phospholipids were extracted from the homogenized tissues
by the method of Bligh and Dyer (22) and lipid phosphorus
determined according to Ames and Dubin (23). Disaturated

phosphatidylcholine in lung lavage was extracted by the method
of Bligh and Dyer (22), separated by the method of Mason *et al.*
(24) and measured by a modification of the enzymatic method
of Muneshighe *et al.* (25), using 3,5-dichloro-2-hydroxybenze-
nesulfonic acid (Aldrich Chemie, Steinheim, W. Germany) as
dye reagent. To assess the recovery, 0.01 μCi [^{3}H]dipalmitoyl-
phosphatidylcholine tracer (New England Nuclear, Boston, MA)
was added to each sample before extraction.

Histology and electron microscopy. At the end of the period of
artificial ventilation, three fetuses selected at random from each
treatment group were studied for lung morphology. The mean
lung-thorax compliance of these fetuses fell within ±0.2 SD of
the means shown in Figure 1. The thorax was opened and the
lung inflated with air to 20 cm H_2O. The pressure was decreased
to 10 cm H_2O while the lungs were perfused for 15 min via the
pulmonary artery with a mixture of 1% glutaraldehyde and 3.5%
formaldehyde in phosphate buffer, pH 7.3. The perfused lungs
were excised and stored in the same fixative until processing.
The paraffin sections of the lungs were oriented from apex to
base and 5-μm sections taken anterior to posterior. After hema-
toxylin-eosin staining, 10 randomly selected fields were point-
counted from apex to base on multiple sections. A field was
rejected if it contained bronchi or large vessels. A standard
morphometric method (26) was used to determine ratios of lung
interstitium to air space. Two lungs from a glucose plus BM-
treated male and a female and two lungs from an inositol plus
BM-treated male and female were further processed for electron
microscopy. The tissues were postfixed with aqueous osmium
tetroxide (2.0% wt/vol), then dehydrated with increasing concen-
trations of acetone, and embedded in Epon. The thin sections
were mounted on copper grids, stained with uranyl acetate, and
lead citrate, and then examined in an electron microscope.
Electron micrographs of type II alveolar cells, visualized in
sections passing through the nucleus and basal and luminal sides
of the cell, were photographed at 8500× magnification. The areas
of glycogen patches were estimated by point counting. The cell
surface and lamellar body areas were measured with an image
analyzer (Kontron, Munich, FRG).

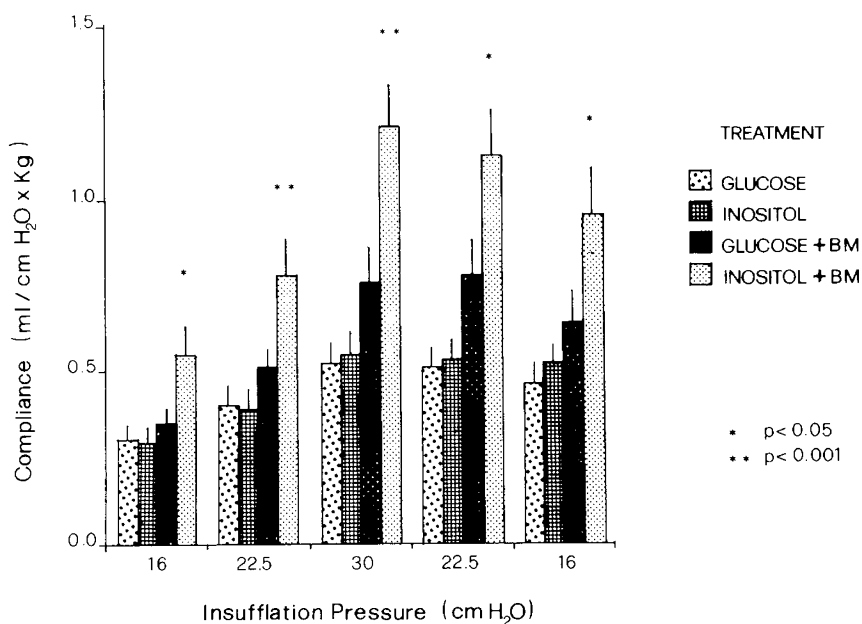*Statistical analysis.* Data were analyzed using the BMPD

Fig. 1. Lung-thorax compliance (ml/cm H_2O × kg) of the four different treatment groups. Animals were ventilated for a total period of 5 min
after birth at insufflation pressures changing every minute from low to high and back to low again (see "Materials and Methods"). Pressures used
are shown on the abscissa from the left following the same chronologic order. Each *bar* represents a different treatment. Values are expressed as
means ± SE. *Asterisks* indicate statistical significance between inositol + BM *versus* glucose ± BM.

(Biomedical Data Processing) statistical software (University of California press, Berkeley, CA) programs 7D and 4V. The results, unless otherwise stated, were expressed as means ± SD. Unless otherwise indicated, the difference between the various treatment groups was evaluated using two-way analysis of variance. The significance of the difference between individual groups was studied using Tukey Studentized range method.

RESULTS

The 31 pregnant rabbits had altogether 164 live fetuses. There was no difference in litter size between the four treatment groups.

Serum inositol. Inositol supplementation increased serum inositol both in does (glucose + saline: 89.6 ± 37.9 μM, $n = 3$; inositol + saline: 295.5 ± 127.0 μM, $n = 3$; glucose + BM: 46.8 ± 18.7 μM, $n = 4$; inositol + BM: 183 ± 157.0 μM, $n = 4$) and in fetuses (glucose + saline: 240 ± 78 μM, $n = 8$; inositol + saline 706.0 ± 202.0 μM, $n = 8$; glucose + BM: 213.4 ± 105.1 μM, $n = 19$; inositol + BM: 459.8 ± 342.0 μM, $n = 27$). At least one fetus in each litter was analyzed. There were no detectable sex differences in serum inositol. The glucose given to the does did not affect either the inositol or the glucose levels (data not shown).

Lung-thorax compliance. To avoid overdistension of the lungs, measurements were made using a positive end expiratory pressure of zero. When inspiratory pressure was changed from low (16, 22.5 cm H_2O) to high (30 cm H_2O), and back to low (22.5 and 16 cm H_2O) again, there was a good correlation between each five consecutive values of lung thorax compliance ($r > 0.90$) (Table 1). In all groups the compliance increased when the insufflation pressure was increased from 16 to 30 cm H_2O (Fig. 1). The compliances observed during the latter part of the ventilation with declining insufflation pressures were higher than the compliances recorded in the early part of the ventilation period, when the insufflation pressure was increasing ($p < 0.02$). This increase in compliance was higher in the BM-treated fetuses (63 ± 3%) than in the controls (48 ± 3%, $p < 0.05$, rank sum test). The order of delivery had no effect on compliance values.

According to variance analysis, BM ± inositol ($p < 0.01$) and inositol ± BM ($p < 0.05$) had a significant effect on compliance (Table 2). Additionally, there was a significant interaction between the effects of BM and inositol ($p < 0.05$). Both the individual five and the mean of these five compliances gave similar results. The effects of inositol ($p = 0.01$), BM ($p < 0.0001$), and their interaction ($p = 0.02$) were highest at the insufflation pressure of 30 cm H_2O. As shown in Figure 1, inositol alone had no effect on compliance. However, when given together with BM, inositol significantly increased the compliance ($p < 0.001$). BM alone tended to increase the compliance as compared to the nonglucocorticoid groups ($p = 0.05$) (Table 3).

The absolute values of lung-thorax compliance (ml/cm H_2O) were analyzed. According to these figures, inositol + BM significantly increased compliance ($p < 0.05$), whereas saline + BM had no significant effect (data not shown).

Effect of fetal sex. We also evaluated lung-thorax compliance/kg wt for possible sex-related difference. In the absence of inositol supplementation, the individual means of the compliances were higher for females than for males. However, the difference between the sexes was not statistically significant. Inasmuch as females that are located between two male fetuses *in utero* develop similarly to males (12), these female fetuses were excluded in the following analysis. Table 1 shows the lung-thorax compliance of the four groups divided by sex, at an insufflation pressure of 30 cm H_2O. In males treated with inositol + BM, the compliance was higher than in any other group, whereas in females there was no detectable difference between inositol + BM and glucose + BM-treated animals. Multivariate analysis of compliances revealed no interaction between sex and the glucocorticoid (± inositol) effect ($p = 0.78$). However, the interaction between sex and the inositol (±BM) effect was almost significant ($p = 0.06$). Variance analysis using inositol treatment (±BM) and sex as dependent variables revealed a significant interaction between treatment and sex ($p = 0.04$). According to the Duncan multiple range test, the inositol treated males had a higher mean compliance than the glucose-treated males ($p = 0.05$).

Lung lavage DPC. The effect of inositol on lavageable surfactant pools was studied by measuring the DPC content of lung lavage fluids. As shown in Table 2, inositol plus BM increased lavageable DPC in fetuses that had not breathed. At the end of the artificial ventilation, no difference was observed in lung

Table 2. *Effects of inositol and BM on amount of disaturated phosphatidylcholine (DPC) in lung lavage at birth and after artificial ventilation**

Treatment	1 Glucose + saline	2 Inositol + saline	3 Glucose + BM	4 Inositol + BM
Lung lavage DPC (nmol/animal)				
At birth	21 ± 8 (7)	24 ± 7 (7)	28 ± 7 (9)	40 ± 1† (10)
After artificial ventilation	28 ± 11 (6)	27 ± 4 (6)	56 ± 20‡ (9)	59 ± 25§ (8)

* Values are means ± SD for number of determinations in parentheses.
† $p < 0.05$ versus 2 and 3.
‡ $p < 0.05$ versus 1.
§ $p < 0.05$ versus 2.

Table 1. *Lung-thorax compliance (ml/cm H_2O × kg) at insufflation pressure of 30 cm H_2O; influence of sex**

Treatment	1 Glucose + saline	2 Inositol + saline	3 Glucose + BM	4 Inositol + BM
Males	0.47 ± 0.26 (13)	0.60 ± 0.23 (9)	0.73 ± 0.42 (13)	1.30 ± 0.57†‡ (13)
Females	0.57 ± 0.31 (11)	0.50 ± 0.27 (11)	0.84 ± 0.47 (13)	1.10 ± 0.59‡ (11)
Females, those between males excluded	0.60 ± 0.24 (8)	0.45 ± 0.21 (9)	0.95 ± 0.39§ (11)	1.10 ± 0.59‡ (11)

* Values are expressed as means ± SD for number of animals in parentheses.
† $p < 0.05$ versus 3.
‡ $p < 0.02$ versus 1 and 2.
§ $p < 0.05$ versus 2.

lavage DPC between fetuses treated with glucose plus BM and with inositol plus BM. In both groups, however, the lavageable pools were higher than in non-BM-treated fetuses ($p < 0.05$).

Body weight and lung growth. As shown in Table 3, BM significantly decreased fetal body weight. There was a concomitant decrease in total lung DNA and phospholipid. However, inositol supplementation partially prevented the BM-induced decrease in lung protein. Inasmuch as total BW was lower in the BM-treated fetuses, lung proteins, DNA, and phospholipid were adjusted for BW. The lung protein/g BW was higher ($p < 0.01$) in fetuses treated with inositol plus BM not only with respect to glucose plus BM, but also when compared to glucose plus saline-treated controls. Inositol ± BM increased the DNA content/g BW when compared with glucose plus saline-treated animals ($p < 0.01$).

Lung morphology. The lungs from saline (± inositol)-treated animals had prominent interstitium; this was associated with localized nonhomogeneous aeration. However, lungs from glucocorticoid-treated animals, especially those that additionally were exposed to inositol, revealed homogenous aeration and wide air spaces. Morphometric analysis showed the following interstitium-to-air-space rations: group 1: 1.3 ± 0.4; group 2: 1.2 ± 0.5: group 3: 0.82 ± 0.30 ($p < 0.05$ *versus* 1); group 4: 0.48 ± 0.20 ($p < 0.05$ *versus* 1, 2, and 3).

Altogether 80 randomly selected type II cells were studied. There was no difference in the number of cells in sections of lungs from fetuses of rabbits treated with inositol + BM and of animals treated with glucose ± BM. Nor was there a detectable difference in the area occupied by type II cells, or the percentage area occupied by glycogen patches within the type II cells (Table 4). Within a single type II cell, however, both the number and the area occupied by lamellar bodies were significantly higher in lungs from fetuses treated with inositol + BM.

DISCUSSION

The present study indicates that dietary inositol modifies the physiologic and biochemical response of the immature fetal lung to pharmacologic doses of exogenous glucocorticoids. In the absence of BM, inositol supplementation at least doubled fetal serum inositol, but failed to improve the lung-thorax compliance. Glucocorticoid given alone resulted in a barely detectable increase in the compliance. However, when given together, inositol and BM produced a clear-cut improvement in compliance and decreased interstitium-to-air space ratios, suggesting homogenous expansion of alveoli.

There are several possible mechanisms that explain the ob-

served synergism between glucocorticoid and inositol. Exogenous glucocorticoid decreased the energy-independent uptake of inositol into the fetal lung (27). Therefore it is possible that the extra inositol is required to offset the glucocorticoid-dependent decrease in inositol uptake in immature lung. The suggested functions of inositol include stimulation of the glucose-6-phosphate dehydrogenase pathway (19), which provides the NADPH required for endogenous fatty acid synthesis (28). Furthermore, inositol is rate-limiting for the synthesis of phosphatidylinositol, which may activate CTP phosphocholine cytidylyltransferase, an enzyme limiting the rate of surfactant phosphatidylcholine synthesis (29). Inositol additionally may activate of the phosphoinositide cycle and protein kinase C (30), known to promote growth and differentiation (31).

According to recent evidence, the improved lung stability after glucocorticoid was not associated with an increase in the lavageable surfactant pool (32, but see Ref. 33). Additional factors, other than surfactant, may influence neonatal lung stability. These include alveolar capillary permeability (34), composition and microstructure of alveolar surfactant (35) or the connective tissue of the lung (36–38). In our study, inositol + glucocorticoid increased lavageable surfactant in the fetus. However, after the brief period of ventilation, there were no detectable differences in surfactant pools between the two groups of glucocorticoid-treated animals (Table 2), despite a consistent difference in lung-thorax compliance. However, the number of lamellar bodies in the type II cells of fetuses treated with inositol + glucocorticoid and ventilated tended to be higher than after glucose plus glu-

Table 4. *Morphometric analysis of type II cells*

Treatment	Glucose + BM ($n = 42$)	Inositol + BM ($n = 38$)
Cell surface area (μm^2)	64.7 ± 2.4	65.1 ± 2.1
Area of lamellar bodies/cell (μm^2)	1.8 ± 0.7	2.6 ± 0.6*
Area of glycogen patches/ cell (μm^2)	14.7 ± 4.9	12.8 ± 4.4
No. of lamellar bodies/section of cell	3.4 ± 0.7	5.3 ± 1.3†

* $p < 0.05$ as compared with glucose + BM.
† $p < 0.01$ as compared with glucose + BM.

Table 3. *Effects of inositol and BM on BW and total lung proteins, phospholipids, and DNA**

Treatment	1 Glucose + saline	2 Inositol + saline	3 Glucose + BM	4 Inositol + BM
BW (g)	38.7 ± 6.4 (34)	35.4 ± 5.6 (33)	31.4 ± 4.1† (49)	31.9 ± 3.1† (47)
Lung proteins (mg)	72.1 ± 13.8 (34)	73.0 ± 14.9 (33)	60.5 ± 10.9‡ (39)	67.4 ± 13.2 (36)
Lung DNA (mg)	4.5 ± 0.7 (24)	5.0 ± 0.8 (26)	4.2 ± 0.7‡ (35)	4.1 ± 0.6‡ (35)
Lung phospholipid (μmol)	13.5 ± 3.0 (33)	13.0 ± 4.2 (36)	11.3 ± 1.7§ (36)	11.2 ± 1.9§ (35)

* Values are means ± SD for numbers of observations in parentheses.
† $p < 0.01$ *versus* 1 and 2.
‡ $p < 0.05$ *versus* 1 and 2.
§ $p < 0.01$ *versus* 1.

cocorticoid, suggesting an increase in intracellular surfactant and/or a decrease in ventilation-induced exocytosis of surfactant. The possibility that the hormone-inositol interaction produced a decrease in alveolar-capillary permeability, or a change in lung connective tissue, remains to be studied.

In vivo studies have indicated that the response to exogenous glucocorticoids is sex-specific, with females responding better than males (6, 39), and that the phenomenon is androgen dependent (12). According to our results, inositol supplementation reversed the so-called "male disadvantage" in fetal lung maturation, since the inositol treatment of males tended to increase the compliance whereas this failed to occur in females (Table 1). Although intriguing, the mechanism of action of inositol, possibly counteracting the negative effect of androgens, remains elusive. As an approach to studying the mechanism of sexual dimorphism, avian fetuses with reversed sex karyotypes were found to have a male instead of a female advantage in the sex-specific response to glucocorticoid and androgen (12). The lack of surfactant phosphatidylglycerol even in an adult avian species (40) suggests exceptionally high alveolar inositol. There are population-based differences found in human sexual dimorphism of fetal lung maturation (6, 7). Therefore, the possibility that inositol is a determinant of the sex difference in surfactant synthesis deserves further consideration.

According to the present (Table 3) and the previous study (19), inositol modified the abnormal glucocorticoid-induced pattern of fetal lung growth toward normal. However, it failed to correct for the decrease in body weight or the decrease in lung DNA and total lung phospholipid after glucocorticoid. Besides inositol, there are other nutrients, growth factors, and hormones (8, 9) that modify the glucocorticoid-induced perturbation in lung growth and the glucocorticoid-induced alteration in the timing of lung differentiation. A more complete understanding of the underlying mechanisms is required to maximize the effectiveness and safety of glucocorticoid in preventing RDS.

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