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# A Rabbit Model for Optimization of Amniotic Fluid Components in the EXTrauterine Environment for Newborn Development (EXTEND) System

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**Keywords**
Amniotic fluid · Animal model · Fetal growth and development · Fetal rabbit

**Abstract**
In this model article, we present a protocol for continuous amniotic fluid exchange in rabbits using a novel system to test the effects of growth factor-deficient, artificial amniotic fluid on bowel development. ***Background:*** Ideally, the EXTrauterine Environment for Neonatal Development (EXTEND) will provide physiologic support to the extreme premature infant. An important component of that environment is the amniotic fluid. Thus, we developed an animal model to study the growth factors found within amniotic fluid and inform design of a synthetic fluid to optimize fetal development. ***Methods:*** We designed a model of amniotic fluid exchange within the pregnant rabbit, continuously removing the natural fluid from around 2 fetuses per doe and replacing it with a physiologic electrolyte solution during the final 100 h of gestation. Two fetuses from the contralateral uterine horn were used as sham-operated controls. Thirty-eight fetuses were analyzed, 19 in each group. We analyzed the fetal growth and bowel development. ***Results:*** Ultrasound after 100 h of exchange showed equivalent fluid volumes, $p = 0.63$. Cultures were negative for bacterial colonization. Final fluid protein concentrations were 11.6% that of control fluid (mean 1,451 ± 224.2 vs. 12,491 ± 849.2 µg/mL). There was no significant difference in fetal growth, with experimental weights 91.4% of control weights, $p = 0.07$. Fetal bowel weights (90.1%, $p = 0.16$) and lengths (94.2%, $p = 0.49$) were also not significantly less compared to controls. There was no significant difference in villous height or crypt depth measurements between the groups, and absorptive capacity of the bowel was not different between groups, $p = 0.44$. ***Conclusion:*** This animal model allows for manipulation of the components of amniotic fluid. Marked reduction of natural amniotic fluid proteins during gestation does not appear to significantly impair fetal growth or bowel development. Further work with this model will assess the importance of amniotic fluid components for normal development to inform design of a synthetic fluid for use during EXTEND.



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## Introduction

Despite extensive research and a variety of interventions and preventive strategies, the rate of preterm birth has steadily increased over the past 20 years [1]. Over one-third of all infant deaths in the USA [2, 3] are attributed to extreme prematurity, making prematurity the leading cause of neonatal death [1]. To reduce morbidity and mortality associated with prematurity, we developed the EXTrauterine Environment for Neonatal Development (EXTEND), aimed at limiting iatrogenic injury to immature organ systems [4]. The system incorporates a pumpless oxygenator circuit connected to the fetus via an umbilical cord interface that is maintained within a closed "amniotic fluid" environment that closely reproduces the environment of the womb. Fetal lambs maintained within this system for up to 4 weeks demonstrate stable hemodynamics, normal fetal blood gas and oxygenation parameters, normal fetal circulation, and normal lung maturation, brain growth, and myelination [4–7].

The "amniotic fluid" used in the system is a physiologic electrolyte solution devoid of natural amniotic fluid growth factors. There are several components of amniotic fluid that have been shown to influence development. Despite the absence of these factors in the EXTEND system, we have found, through thorough evaluation of intestinal maturation within EXTEND, that the bowel does continue to mature and is comparable to age-matched control bowel (Baumgarten et al., unpublished results). However, we still seek to optimize the fluid used within EXTEND to support fetal development.

Here, we present a novel animal model directed toward screening and identification of important amniotic fluid components required for optimal fetal growth and development. This is a model for continuous amniotic fluid exchange around the fetal rabbit. Although there have been several studies looking at amniotic fluid additives during late gestation in the fetal rabbit [8–12], continuous amniotic fluid replacement has not been published and will allow systematic evaluation of the effects of various swallowed elements on fetal development, absent the influence of the full normal complement of amniotic fluid factors. We hypothesized that the depleted amniotic fluid might result in impaired growth for experimental fetuses.

## Materials and Methods

*Animals*

This study protocol was approved by the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee. The average gestation for the rabbit is 31 days; hence, we started each study at 25 days gestation, allowing for 100 h of continuous amniotic fluid replacement. We used gestation-day time-dated New Zealand white female rabbits, weighing approximately 3.5 kg, for the experiment. On average, there are 4–6 pups in each uterine horn. Acclimation prior to surgery was performed to allow for familiarity with the jacket, weight of the backpack contents, and neck cone, ensuring adequate mobility within the cage. Nineteen experimental fetuses and 19 control fetuses are included in this proof-of-concept study. The fluid used for this study was the same as that currently used within EXTEND – a "physiologic saline solution" (92 mEq NaCl, 19.4 mEq $NaHCO_3$, 6.7 mEq KCl, and 1.7 mEq $CaCl_2$, pH 7.0) without any growth factor additives.

*Materials*

1. 7-Fr dual lumen silicon catheters (Cook Medical, cat#G08693) are used for infusion and drainage of fluid. Catheter length is trimmed to approximately 22 cm. Three additional holes are placed along the final 2 cm of the larger lumen that will become the drainage side of the catheter. A small donut or ring is made out of pledget material and glued around the catheter 2.5 cm from the end of the catheter. This helps bolster the amniotic membranes to the insertion site and decrease amniotic membrane separation. Silicone is used to occlude the terminal end of the large lumen to promote adequate circulation of infused fluid. These are sterilized with ethylene oxide.
2. Low-pressure ventriculoperitoneal shunt valves (Medtronic, cat#42412) are used to maintain a physiologic intra--amniotic pressure between 3 and 5 mm Hg. These are placed in line with the drainage port of the catheter prior to connection with the drainage reservoir.
3. The 400-mL dual site On-Q pain infusion pump (Halyard, model#CB4007, part#103161400) is used to infuse the experimental fluid into 2 amniotic spaces. This pump allows for infusion of fluid at a rate of 1–7 mL/h. The infusion pump is set to a rate of 7 mL/h.
4. Jackson-Prattbulbreservoirs(CardinalHealth,cat#SU130-1305) are used to collect the drainage fluid.
5. Rabbit jackets and pouches (SAI Infusion Technologies, cat#BJ03CUST and JP11) are used to carry the system. The pouch is attached to the jacket with zip ties. A hole is cut in the jacket to allow for the catheters to pass from the flank of the doe into the pouch.

*Creation of the Continuous Amniotic Fluid Exchange Model*

On the 25th day of gestation, the doe is prepared for surgery, beginning with intramuscular injection of ketamine (20–50 mg/kg) and xylazine (2.5–5 mg/kg). Isoflurane inhalant is administered via facemask for induction of anesthesia, and the rabbit is intubated using a 3- or 3.5-Fr endotracheal tube. DepoProvera (40,000 U/kg administered intramuscular) is given to prevent preterm delivery. Buprenorphine (0.01–0.05 mg/kg) is given subcutaneously prior to incision, and a fentanyl patch is placed over a shaved area on the scapula. Propofol is used intermittently throughout surgery to decrease uterine contractility.

The abdomen of the doe is prepped with Betadine and draped sterilely. Ioban is used to cover the operative field to improve sterility. A lower midline incision is made, and care is taken to separate the mammary tissue down the midline without injury. A hollow tunneling tool is used to create a tract from the midline to the left flank, and the catheters are introduced in an antegrade fashion. The abdomen is then opened and one uterine horn externalized. The first and third fetuses are used in the experiment. The first in the horn is the farthest from the cervix and the third is in the position of natural growth restriction [11] in the rabbit (Fig. 1). The corresponding kits in the contralateral horn serve as controls [11, 12].

Fetus 1 is identified. A pocket of fluid is created over the center of the fetus on the anti-mesenteric side of the uterus to avoid the placenta and prevent traversing 2 fetal cavities upon catheter placement. Ultrasound is then used to confirm appropriate placement location and adequate space between entry site and fetus. Three stay sutures are placed around the planned entry site using 5-0 prolene. These are meant to secure the amniotic membrane to the uterine muscle and then gently tent these layers up together during catheter placement. A purse string suture is placed around the stay sutures, using 5-0 prolene. Hysterotomy is made with an 11-blade. Jacobson micro-mosquito forceps are used to dilate the opening without disrupting the internal membranes. At this time, an initial fluid sample can be collected.

The catheter is placed through the hysterotomy and the purse string is pulled snug against the catheter. The catheter is retracted to approximate the pledgeted portion of the catheter against the hysterotomy site. The stay sutures are removed, and the purse string is tied down. Finally, a 2-0 silk suture is wrapped around the insertion site and tied down, ensuring a rim of uterine tissue is encircled.

The On-Q pump is filled with 180 mL of experimental fluid and connected to the small lumen of the dual lumen catheter after ensuring the line is clear of any air. Three milliliters of fluid is infused to replace the fluid lost upon catheter placement. The pressure valve is connected to the drainage (large) lumen of the catheter. These steps are repeated for the third fetus in the ipsilateral uterine horn. The uterus is then reduced into the abdominal cavity ensuring no torsion, and the abdomen is closed in a standard 2-layer fashion. The catheters are secured at their exit site on the left flank. Skin glue is used to seal the abdominal incision and the catheter exit sites.

*Postoperative Care*

The abdomen of the doe is wrapped with Kerlix gauze and cotton undercast padding. This dressing is secured with self-adherent bandaging tape. This wrap helps prevent seroma formation and infection and is removed on postoperative day 2. The rabbit jacket is then placed, system contents organized into the pouch, and pouch secured to the rabbit jacket. A neck cone is placed prior to returning the doe to her cage to keep her from chewing the catheter insertion and incision sites. During recovery from anesthesia, the system needs frequent flushing to ensure no air is retained within the system. Throughout the 100-h experiment, the system should be checked and flushed every 3 h. Maintaining sterile technique when accessing the system is important. For this study, drainage fluid was collected every 6 h for protein concentration measurement. The infusion pump is refilled to a maximum of 180 mL twice daily.

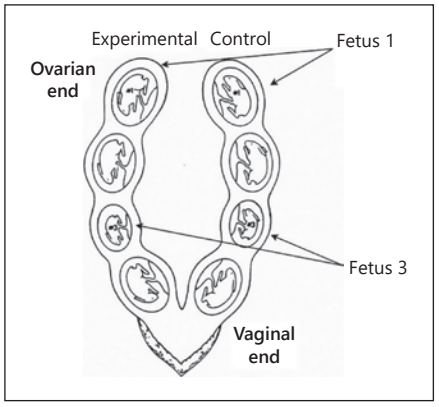


**Fig. 1.** Rabbit anatomy. *Image taken from publication by Flake AW, et al. Transamniotic fetal feeding. J Pediatr Surg. 1986.* This study utilized fetuses in the first and third positions of one uterine horn. Those fetuses from the contralateral uterine horn were used as controls. The third fetal position in rabbits has impaired blood supply, resulting in intrauterine growth restriction, making that fetus, perhaps, more sensitive to changes in the amniotic fluid environment.

*Necropsy*

After 100 h, the doe is taken for terminal surgery. The doe is not intubated for this procedure. She is monitored with a pulse oximeter and rectal temperature probe. The jacket is removed and system disconnected from the catheters. The drainage in the bulb reservoirs is collected for analysis. The abdomen is prepped and draped in usual sterile technique. The midline laparotomy is reopened and the experimental uterine horn eviscerated. Ultrasound is used at this point to measure amniotic fluid volume around the experimental fetuses and around the contralateral uterine horn control fetuses. A hysterotomy is then made over the head of experimental fetus 1. A culture swab is inserted into the cavity, and a sample of amniotic fluid is collected using a 20-μL pipette. The fetus is then positioned so the mouth is accessible through the hysterotomy. An orogastric tube is placed, and 300 μL of 10% galactose solution is administered. The uterus is then closed with a figure-of-eight silk suture, and this fetus is returned to the abdomen. This is repeated for experimental fetus 3 and for control fetuses 1 and 3 on the contralateral uterine horn. Twenty minutes after administering galactose to experimental fetus 1, that fetus is removed from the uterus and decapitated for collection of a blood sample. This is repeated for the remaining fetuses. All 4 fetuses are collected for gross analysis.

The remaining fetuses are euthanized and then the doe is euthanized. Death of the doe is confirmed via thoracotomy.

*Specimen Collection*

Fluid Samples

At the end of each study, bacterial and fungal cultures of the amniotic fluid were sent for evaluation to ensure no contamination of the system (Antech Diagnostics). The final amniotic fluid sample and all drainage samples collected throughout the study are analyzed for protein concentration (Pierce BSA Protein Assay Kit,

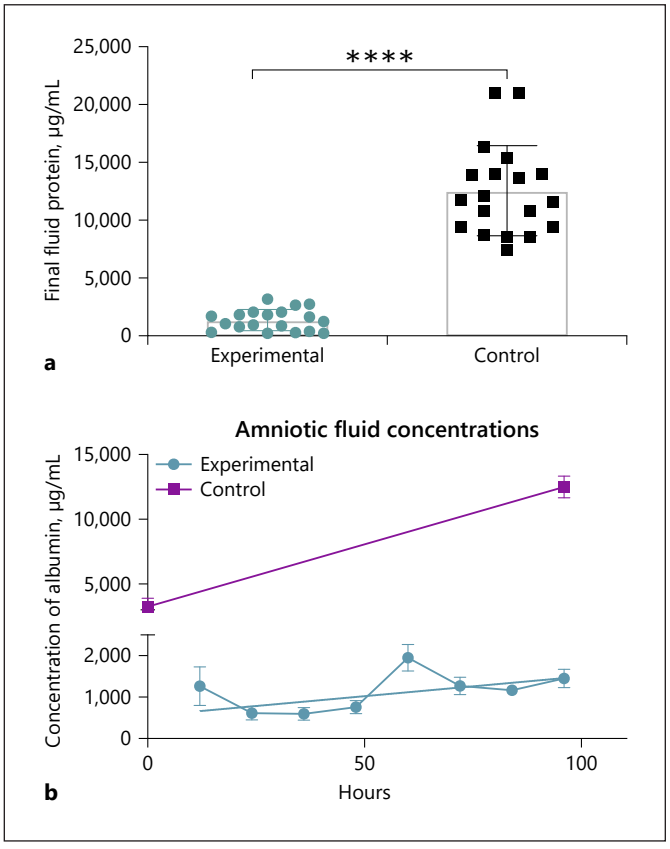


**Fig. 2.** Fluid protein concentration. **a** Final fluid protein concentrations in the experimental group were dilute compared to control fluid. **b** Throughout the experiment, the fluid collected from around the experimental fetuses was dilute compared to controls. As gestation progresses, natural amniotic fluid becomes more protein rich. The protein concentrations of experimental fluid also increased slowly over the course of the experiment.

cat#23225; ThermoFisher Scientific). Fetal serum is analyzed for galactose concentration using a fluorescent assay (cat#MAC012; Sigma-Aldrich).

Gross Tissue Analysis and Histology

Fetal weights and bowel weights, from pylorus to sigmoid colon, were measured. Bowel length was measured using Fiji ImageJ 2.0, freehand line tool. Terminal ileum and proximal colon were formalin-fixed, paraffin-embedded, sectioned at 4 µm, and stained with hematoxylin and eosin for morphologic evaluation [13]. Morphologic analysis of villus height and crypt depth used 2 slides per animal, each with 2–3 transverse sections. Twenty fully intact villus/crypt pairs per animal were measured using the caliper tool within ImageScope Aperio eSlide Manager Version 12.2.1.5005 (Leica Biosystems, Buffalo Grove, IL, USA).

*Statistical Analysis*

Statistical analyses were performed using GraphPad Prism (GraphPad Software). Unpaired Student's *t* tests compared nor-

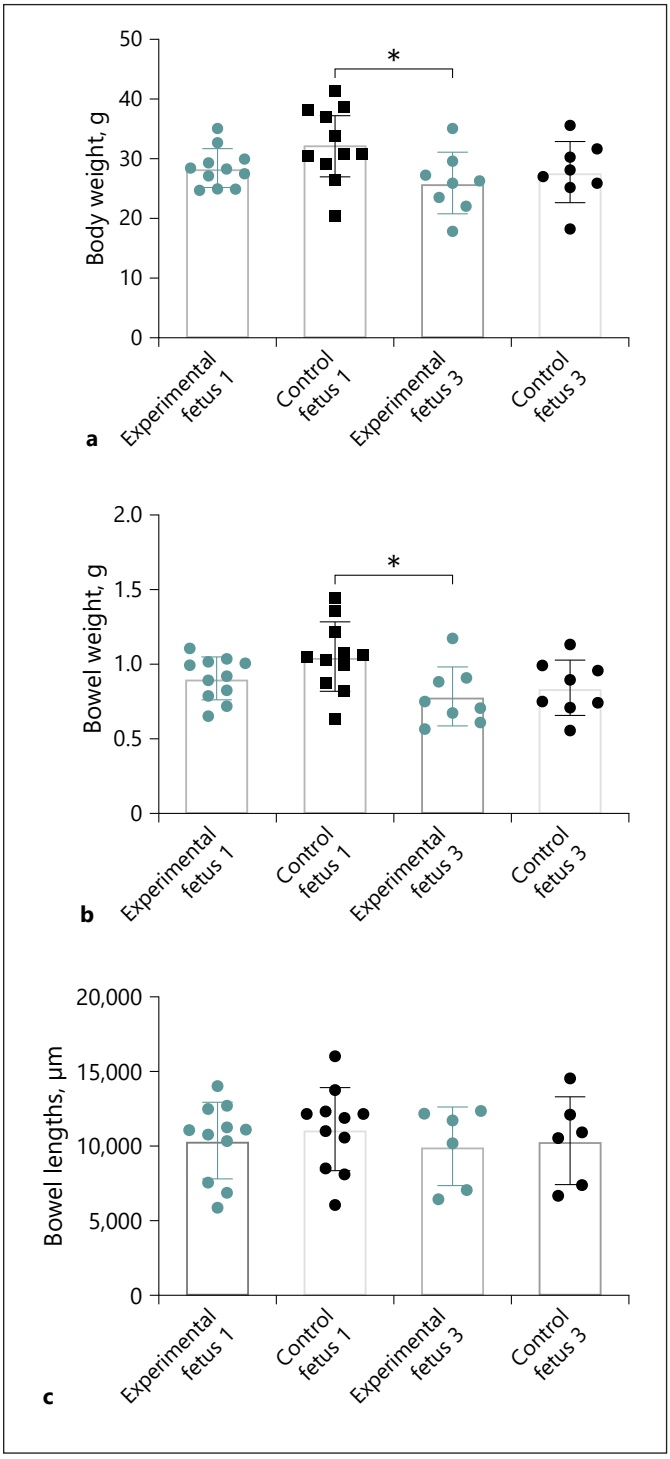


**Fig. 3.** Growth. Fetal weight (**a**), bowel weight (**b**), and bowel length (**c**); measured from the pylorus to the sigmoid colon.

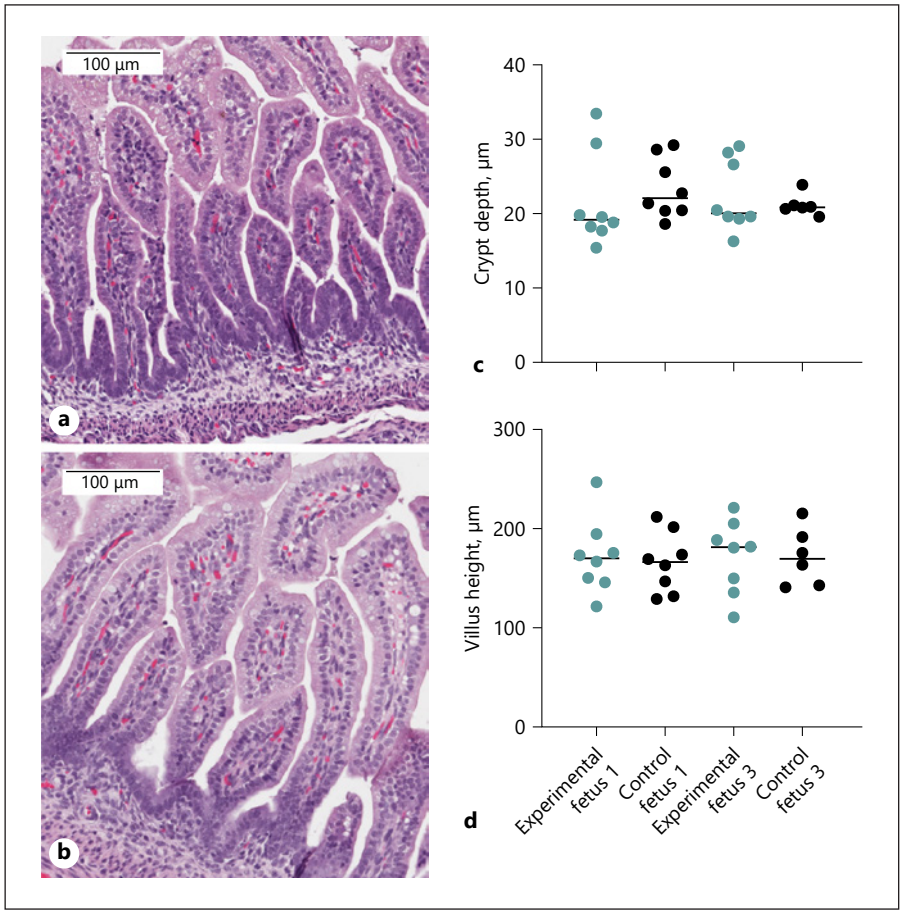


**Fig. 4.** Bowel morphology. H&E-stained ileum from a control fetus 3 (**a**) and an experimental fetus 3 (**b**), villus height (**c**), and crypt depth (**d**).

mally distributed data and are presented as mean ± standard error of the mean (SEM). One-way ANOVA with multiple comparisons was used to evaluate differences between multiple groups. Nonlinear regression analysis was performed to represent fluid concentrations over time.

## Results

*Proof of a Functional Model*

There were no positive culture results. Protein concentration of the final amniotic fluid collected was 11.6% of the control fluid concentration (mean 1,450 ± 224.2 vs. 12,491 ± 849.2 µg/mL, $p < 0.0001$) (Fig. 2a). The slope of the best-fit line representing experimental fluid concentrations throughout the study is 9.49 with *y*-intercept 548.5 compared to 96.3 with *y*-intercept 3,247 for control fluid concentrations from initial to final fluid collections, $p < 0.0001$ (Fig. 2b). Amniotic fluid volume measurements were not different between groups. Single deepest pocket measurements (experimental mean 0.65 ± 0.06 cm vs. control 0.60 ± 0.08 cm, $p = 0.63$) and volume of the largest fluid pocket (experimental mean 0.32 ± 0.03 $cm^2$ vs. control 0.29 ± 0.04 $cm^2$, $p = 0.61$) were both comparable between groups.

*Fetal Growth*

Fetal weights were not significantly different between groups (experimental weight average 28.75 ± 1.02 g vs. control 31.98 ± 1.46 g, $p = 0.08$). When the groups were separated by fetal position, experimental and control weights were still not significantly different (fetus 1, $p = 0.26$; fetus 3, $p = 0.89$); however, there was a significant difference between fetus 1 controls and fetus 3 experimental animals, $p = 0.04$ (Fig. 3a).

Fetal bowel weights were also not significantly different between groups overall (average experimental weight 0.87 ± 0.04 g vs. control 0.98 ± 0.05 g). Similarly, when fetuses 1 and 3 were analyzed separately, the only significant difference was found between control fetus 1 and experimental fetus 3, $p = 0.03$ (fetus 1, $p = 0.30$; fetus 3,

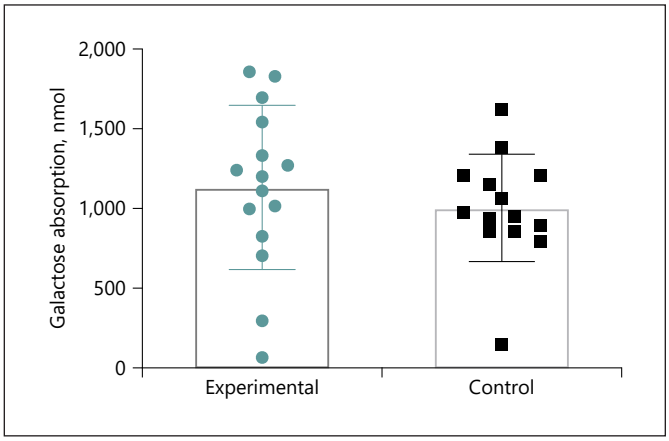


**Fig. 5.** Galactose absorption.

$p = 0.93$) (Fig. 3b). Bowel lengths were also similar (average experimental bowel length 34.1 ± 2.0 vs. 36.22 ± 2.2 cm, $p = 0.49$). One-way ANOVA of the 4 groups, when control and experimental fetus 3 animals were separated into separate groups did not reveal any differences between groups, $p = 0.84$ (Fig. 3)c.

*Bowel Development*

Measurements of villus height and crypt depth were not different between groups (Fig. 4a–d). Average villus height for experimental fetus 1 was 171.9 ± 13.3 versus 166 ± 10.7 μm for control fetus 1, $p = 0.98$; and for experimental fetus 3 was 171.7 ± 13 versus 171.6 ± 11.8 μm for control fetus 3, $p > 0.99$ (Fig. 4c). Crypt depth averaged 21.6 ± 2.2 μm for experimental fetus 1 versus 23.4 ± 1.4 μm for control fetus 1, $p = 0.86$; and for experimental fetus 3, crypts measured 22.4 ± 1.7 versus 21.2 ± 0.56 μm for control fetus 3, $p = 0.96$ (Fig. 4d). Absorptive capacity was also similar between groups. Experimental fetuses absorbed an average of 3,866 ± 413.6 ng/μL of galactose and control fetuses absorbed 3,605 ± 324.6 ng/μL at 20 min (Fig. 5).

## Discussion

Several published studies have used animal models to evaluate the effect of amniotic fluid additives on fetal development issues, such as intrauterine growth restriction and necrotizing enterocolitis (NEC). These previously reported models were used for continuous infusion of fetal intragastric amniotic fluid [14] and galactose [10] or intra-amniotic nutrient solution [11]. However, no previous model has achieved clearance of the full complement of natural fluid factors, confounding the effect of any added substance. This was not a simple task. Yet, we felt it important to successfully eliminate this confounding effect in order to truly evaluate the influence of each growth factor of interest.

The normal volume of amniotic fluid in the rabbit has been reported to range from 1.7 to 4 mL [15, 16]. Complete amniotic fluid turnover once every 24 h [17] is well understood in humans. Within this model, the infusion rate of synthetic fluid is supraphysiologic to avoid accumulation of fetal urine and other factors produced by the placenta and released into the amnion continuously. This necessitates consistent drainage of fluid to maintain the appropriate volume of fluid around the fetus at any given time. Were the drainage system to malfunction – specifically, the ventriculoperitoneal shunt valve, which will not function if there is air in the chamber – the fetal cavity would quickly overfill and occlude blood supply to the fetus, resulting in fetal demise. Frequent system checks and shunt flushing prevents this complication.

This model allows for unrestricted movement of the doe, in contrast to previously reported models using cannulas, tunneled to the nape of the neck and connected to an infusion swivel [14]. This movement is important and also introduces new challenges, particularly related to the balance of the backpack and potential for kinking of the tubing. It was important to acclimate the doe to the backpack prior to surgery, and to identify the optimal volume of fluid to fill the On-Q pump – acceptable weight for optimal mobility was found with the reservoir no fuller than 180 mL. Again, frequent system checks helped identify any shifting of pack contents causing kinked tubing in a timely manner.

As we work to perfect the support provided to the extremely premature infant within the EXTEND system, the ideal composition of the "amniotic fluid" surrounding the neonate is a focus of interest. Although we have not seen a difference in lamb development associated with the absence of natural amniotic fluid trophic factors within EXTEND (Baumgarten et al., unpublished results), and this proof-of-concept study also did not identify any clear differences in fetal rabbit development in the absence of amniotic fluid proteins, this study may be underpowered to see a significant difference between groups. Post hoc power analysis suggests doubling our sample size may result in significant findings in fetal growth measurements. Further, although the analysis of gut development in EXTEND did not show any measur-

able difference between experimental animals and controls – a finding supported by McLeod et al. [18] – much of the existing scientific literature argues for the importance of these proteins, particularly when studied within NEC animal models.

NEC is a leading cause of morbidity and mortality among premature infants in the neonatal intensive care unit (NICU) [19, 20]. The mortality of NEC ranges from 20 to 50% [19, 21–25]. The exact pathophysiology of NEC has not been defined, but prematurity, exaggerated inflammatory response, aberrant bacterial colonization, hypoxia, and intestinal ischemia have all been implicated [19, 22, 25]. During intrauterine development, fetal swallowing of amniotic fluid is thought to stimulate gastrointestinal growth and maturation through growth factors that help regulate mucosal immunity in the prenatal period [26]. Preterm birth interrupts this exposure, possibly contributing to the increased risk of NEC in premature infants [27].

Trophic components of natural amniotic fluid thought to be beneficial for bowel maturation include epidermal growth factor, heparin-binding epidermal growth factor, granulocyte colony-stimulating factor, erythropoietin, tumor necrosis factor beta, various interleukins, and insulin-like growth factor-1, to name a few. In animal models, these factors have all been shown to increase intestinal barrier strength and reduce the severity of experimental NEC [28–31]. These factors modulate the exuberant immune response to intraluminal antigens, and in response to injury, these factors enhance the migration and proliferation of intestinal epithelial cells, leading to a reduction in the severity of intestinal injury. Loss of amniotic fluid trophic factors is hypothesized to predispose to villous atrophy, feeding intolerance, and increased risk of NEC [27] since these swallowed factors provide direct support to gut epithelium. These factors are found in colostrum and breast milk at increasing concentrations in the mothers of the most premature infants [32]. The fetus also receives 10–15% of the nitrogen and energy requirements from swallowed amniotic fluid [26]. Hence, our persistence in pursuit of identifying the factors that might work to enhance continued "fetal" development within EXTEND.

## Conclusion

This novel animal model allows for experimentation with the components of amniotic fluid without the confounding presence of the natural trophic factors within amniotic fluid that are thought to influence fetal development in both overt and subtle ways. In this proof-of-concept study, the marked reduction of natural amniotic fluid proteins during gestation did not appear to significantly impair fetal growth or bowel development. However, these results may represent type II error, and analysis of larger study groups may, in fact, show a negative effect on fetal growth in these conditions. Ultimately, the ideal formula to support fetal development during the late second and early third trimester of gestation is not clearly understood. Further work with this model will focus on elucidating the effects of independent amniotic fluid components on development and will inform intelligent design of an optimal synthetic amniotic fluid for use during EXTEND.


## Acknowledgement

James Moon, MD, continuing the work with this model. Haiying Li, technical support with the galactose assay. Jacqueline Amato, administrative support. Aaron Weilerstein, technical support with animal acquisition and surgery.


## Statement of Ethics

This study does not involve human subjects – only rabbits. Our study complies with the internationally recognized guidelines such as the ARRIVE guidelines. This study protocol was approved by the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee (IACUC); approval number 20-001241.

## Conflict of Interest Statement

The authors have no conflicts of interest to declare.


## Funding Sources

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## Author Contributions

Conception or design of the work: H.D.B., M.D., and A.W.F. Acquisition, analysis, or interpretation of data: H.D.B., H.A.H., Z.B., K.O., A.C.R., K.M.L., A.G.K., M.D., and A.W.F. Drafting the work or revising it critically: H.D.B., H.A.H., Z.B., and A.W.F.

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