Inhibitor of intramembranous absorption in ovine amniotic fluid

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Brace RA, Cheung CY, Anderson DF. Inhibitor of intramembranous absorption in ovine amniotic fluid. Am J Physiol Regul Integr Comp Physiol 306: R185–R189, 2014. First published December 31, 2013; doi:10.1152/ajpregu.00469.2013.—Intramembranous absorption increases during intra-amniotic infusion of physiological saline solutions. The increase may be due partly to the concomitant elevation in fetal urine production as fetal urine contains a stimulator of intramembranous absorption. In this study, we hypothesized that the increase in intramembranous absorption during intra-amniotic infusion is due, in part, to dilution of a nonrenal inhibitor of intramembranous absorption that is present in amniotic fluid. In late-gestation fetal sheep, amniotic fluid volume and the four primary amniotic inflows and outflows were determined over 2-day intervals under three conditions: 1) control conditions when fetal urine entered the amniotic sac, 2) during intra-amniotic infusion of 2 l/day of lactated Ringer solution when urine entered the amniotic sac, and 3) during the same intra-amniotic infusion when fetal urine was continuously replaced with lactated Ringer solution. Amniotic fluid volume, fetal urine production, swallowed volume, and intramembranous absorption rate increased during the infusions independent of fetal urine entry into the amniotic sac or its replacement. Lung liquid secretion rate was unchanged during infusion. Because fetal membrane stretch has been shown not to be involved and because urine replacement did not alter the response, we conclude that the increase in intramembranous absorption that occurs during intra-amniotic infusions is due primarily to dilution of a nonrenal inhibitor of intramembranous absorption that is normally present in amniotic fluid. This result combined with our previous study suggests that a nonrenal inhibitor(s) together with a renal stimulator(s) interact to regulate intramembranous absorption rate and, hence, amniotic fluid volume.

Our understanding of the regulation of amniotic fluid volume has been continually evolving. Current concepts are that, in late gestation, amniotic fluid volume is dependent on a balance between two primary amniotic inflows (fetal urine and lung liquid) and two primary outflows (swallowing and intramembranous absorption). Although fetal urine production, lung liquid secretion, and swallowing have been shown to be regulated by the fetal nervous and endocrine systems, the regulation of intramembranous absorption rate is less well understood.

Conceptually, intramembranous absorption may be regulated by chemical substances present in amniotic fluid and/or stretch of the fetal membranes. However, membrane stretch has been shown to have no effect on amniotic fluid volume in late-gestation sheep (7). The presence of stimulators and/or inhibitors of intramembranous absorption in amniotic fluid was demonstrated in amniotic fluid washout studies (1, 18). In those experiments, lactated Ringer solution was continuously infused into the amniotic compartment at 3 l/day for 3 days, while amniotic fluid was withdrawn at the same rate and discarded. Amniotic fluid volume nearly doubled during washout, suggesting the presence of chemical regulators in amniotic fluid that function to modulate intramembranous absorption (18). Further, the change in amniotic fluid volume during the washout differed between intact fetuses and those with a tracheoesophageal shunt, suggesting either a pulmonary source or removal by swallowing of amniotic fluid regulatory factors of intramembranous absorption that are present in amniotic fluid. Subsequent studies showed that fetal lung liquid secretions contain neither stimulators nor inhibitors of the active or passive components of intramembranous absorption (9, 12, 13).

Other studies have shown that intra-amniotic infusion of water, saline, or lactated Ringer solution leads to increases in amniotic fluid volume and intramembranous absorption rate (5, 10, 11, 13). Although the cause of these increases in intramembranous absorption has not been explored, the fetal kidneys could be involved. Fetal urine contains a stimulator of intramembranous absorption (2) and, with fetal urine flow increasing during the intra-amniotic infusions (10, 13), the kidneys may deliver increased amounts of the intramembranous stimulator into the amniotic fluid. It is also possible that the increase in intramembranous absorption rate during intra-amniotic infusions results from dilution of nonrenal, nonpulmonary inhibitors of intramembranous absorption that are normally present in amniotic fluid. In this study, we explored the role of nonrenal regulators of intramembranous absorption during intra-amniotic fluid infusion when fetal urine entered the amniotic compartment and when fetal urine was continuously replaced with an equal volume of fluid. We hypothesized that the normal increase in intramembranous absorption rate that occurs during intra-amniotic infusion would be reduced but not eliminated by isovolumic replacement of fetal urine.

Methods

Ethical considerations. These studies were approved by the Institutional Animal Care and Use Committee of Oregon Health and Science University. The National Research Council’s Guide for the Care and Use of Laboratory Animals was followed.

Animal preparation. These studies were conducted in five late-gestation, chronically catheterized fetal sheep. Surgical catheterization of the fetus was performed at 119–120 days gestation (term ~ 145 days). Under general anesthesia using aseptic techniques, catheters were placed as previously described in a fetal carotid artery for monitoring fetal blood gas status, in the trachea for measuring lung liquid secretion rate, and in the urinary bladder for measuring urine production (13). The urachus was ligated to prevent urine entry into the allantoic sac. A flow probe (Transonic Systems, Ithaca, NY) was placed around the midcervical esophagus for measuring swallowing (13, 17). Multiple catheters were attached to the fetal skin for access to the amniotic fluid. All incisions were repaired. The flow probe
cable and catheters were tunneled subcutaneously and exited into a pouch on the maternal flank. Prophylactic antibiotics (1 million units penicillin G intramuscularly to the ewe and 2 mg ciprofloxacin into the amniotic fluid) were administered at the end of surgery, and the ewe was given 0.6 mg buprenorphine for 2 days for analgesia.

**Experimental measurements.** At 4 days after surgery, the ewe was brought into the recording laboratory and placed in a cart with free access to food and water. Continuous monitoring of swallowing, urine production, and lung liquid secretion rates was initiated as previously described (13). Experiments began the following day with random assignment to one of three protocols, each lasting 2 days: 1) control with urine entering the amniotic sac; 2) intra-amniotic infusion of lactated Ringer solution at 2 l/day with urine entering the amniotic fluid; and 3) the same intra-amniotic infusion while fetal urine was continuously and isovolumically replaced with lactated Ringer solution (2). Each animal completed all three protocols.

Immediately prior to the beginning each protocol, amniotic fluid volume was determined by drainage (2, 13), and the amniotic fluid was replaced with 1 liter of warm lactated Ringer solution, so that all protocols started with the same volume and composition of amniotic fluid. At the end of each protocol, fetal arterial blood was sampled for blood gases, pH, hematocrit, and oxygen content (Radiometer model 725, corrected to 39°C fetal body temperature), and amniotic fluid volume was measured. Fetal blood and amniotic fluid solute concentrations (potassium, sodium, calcium, chloride, glucose, and lactate) were determined with an automated analyzer (Radiometer model 725).

**Data presentation and statistical analyses.** Data are presented as means ± SE. Values during the different protocols were analyzed with a paired t-test or a two-factor repeated-measures ANOVA. Post hoc testing utilized Fisher’s least significant difference for multiple comparisons when the null hypothesis was rejected. When no differences existed, overall mean values are presented for the three protocols combined. Bivariate linear regression was used to determine whether significant relationships existed between urine flow and intramembranous absorption rates during each of the three protocols separately. A one-factor analysis of covariance (ANCOVA) was used to compare the relationships between intramembranous absorption rate and urine flow rate 1) in individual fetuses during the three protocols and 2) in all fetuses combined under control conditions vs. intra-amniotic infusions with and without urine replacement. Statistical significance was accepted if $P < 0.05$.

**RESULTS**

Amniotic fluid volume averaged 705 ± 245 ml at the end of the control period. Following a 2-day intra-amniotic infusion of 2 l/day of lactated Ringer solution, amniotic fluid volume increased significantly above control volumes ($P = 0.0069$) by 2,308 ± 363 ml when fetal urine entered the amniotic fluid and by 1,931 ± 646 ml above control when urine was isovolumically replaced (Fig. 1). The amniotic fluid volumes during the infusions were not significantly different ($P = 0.56$). Intramembranous absorption rate was 905 ± 111 ml/day during the 2-day control period. Absorption rate increased significantly ($P = 0.011$) to twice normal during the 2 l/day intra-amniotic infusions. The increases were 1,019 ± 283 ml/day above control values when urine entered the amniotic fluid and 1,149 ± 356 ml/day when urine was replaced were not different ($P = 0.65$). Fetal urine flow and swallowing responded similarly, increasing during the infusions, with urine replacement having no significant effect (Fig. 2). Lung liquid secretion rate trended upward during the infusions but did not change significantly. The infused fluids combined with the excess volumes of urine during the 2-day infusions totaled 5,010 ± 282 ml and 4,838 ± 287 ml without and with urine replacement, respectively, or more than twice the increases in amniotic fluid volume.

The relationship between intramembranous absorption rate and urine flow rate is shown in Fig. 3. Although these two
variables were not significantly related \((P > 0.2)\) under each of the three experimental conditions when analyzed separately (Fig. 3, top), there was a significant upward shift in the relationship during intra-amniotic infusion \((P = 0.01)\) that did not depend on the entry of urine into the amniotic fluid. The lower panel of Fig. 3 shows that there were significant differences in the responses of individual fetuses to the infusions. Fetuses with the lowest control urine flows had the greatest increases in intramembranous absorption rate in response to the infusions and vice versa.

Amniotic solute concentrations changed little if at all during the intra-amniotic infusion. Amniotic fluid potassium \((P = 0.91)\) and glucose \((P = 0.42)\) concentrations were unchanged compared with control values, averaging \(5.0 \pm 0.5 \text{ mmol/l}\) and \(0.3 \pm 0.1 \text{ mmol/l}\), respectively, for the three protocols combined with no differences with and without urine replacement. Amniotic sodium and chloride concentrations tended to increase during the infusions compared with control values, but the increases were not significant (Fig. 4). Amniotic calcium and lactate concentrations increased during the infusions (Fig. 4). Overall, mean amniotic sodium \((127.4 \pm 2.6 \text{ mmol/l})\), calcium \((1.20 \pm 0.13 \text{ mmol/l})\), and chloride \((100.8 \pm 1.1 \text{ mmol/l})\) concentrations were similar to their Ringer infusate concentrations (Fig. 4).

Fetal blood potassium \((3.8 \pm 0.1 \text{ mmol/l})\), sodium \((141.0 \pm 0.8 \text{ mmol/l})\), and lactate \((1.6 \pm 0.1 \text{ mmol/l})\) concentrations were unchanged during the intra-amniotic infusions compared with control. Fetal blood calcium \((1.47 \pm 0.06 \text{ mmol/l} vs 1.59 \pm 0.06 \text{ mmol/l})\) and chloride \((104 \pm 1.5 \text{ mmol/l} vs 107 \pm 1.9 \text{ mmol/l})\) concentrations were increased compared with control during intra-amniotic infusion with urine replacement but not when urine entered the amniotic cavity. Fetal blood glucose concentration decreased \((1.12 \pm 0.05 \text{ mmol/l} vs 0.96 \pm 0.08 \text{ mmol/l})\) during the intra-amniotic infusion when urine entered the amniotic fluid but not when urine was replaced.

Intramembranous solute concentration differences (fetal-amniotic) also changed little if at all during the intra-amniotic infusions. The potassium, sodium, calcium, chloride, and glucose concentration differences did not change significantly, averaging \(-0.76 \pm 0.6, 13.7 \pm 4.7, 0.4 \pm 0.1, 3.7 \pm 3.2, \text{ and } 0.7 \pm 0.1 \text{ mmol/l}\), respectively, for the three protocols combined. With the increase in amniotic and unchanged blood lactate concentrations, the intramembranous lactate concentration difference decreased from \(-1.9 \pm 0.2 \text{ mmol/l}\) at the end of the control period to \(-7.7 \pm 1.7\) and \(-8.4 \pm 1.4 \text{ mmol/l}\) during the intra-amniotic infusions without and with urine replacement, respectively.

There were no significant changes among the three protocols when comparing fetal arterial pH \((\text{mean } = 7.36 \pm 0.01)\), carbon dioxide tension \((51.2 \pm 0.9 \text{ mmHg})\), oxygen tension \((22.1 \pm 0.7 \text{ mmHg})\), hematocrit \((31.8\% \pm 1.2\%)\), or blood oxygen content \((7.7 \pm 0.3 \text{ ml/dl})\).

![Fig. 3. ANCOVA comparisons of fetal urine flow rate and intramembranous absorption rate. Upper panel compares control conditions (●) with intra-amniotic infusion of lactated Ringer solution at 2 l/day when urine entered the amniotic sac (●) or when urine was isovolumically replaced (●). Lower panel compares values in each fetus separately for the three protocols. Each fetus is represented by a different symbol and regression line.](http://ajpregu.physiology.org/)

![Fig. 4. Amniotic fluid sodium, chloride, lactate, and calcium concentrations after 2 days of either control conditions, intra-amniotic infusion of 2 l/day of lactated Ringer solution (infusion), or 2 l/day of lactated Ringer solution infusion plus isovolumic replacement of fetal urine (Inf + Repl). Data are means ± SE. Points with no error bars have SE within the dot. \(P\) values are from two-factor repeated-measures ANOVA. *\(P < 0.05\) compared with control from post hoc testing. Dashed lines are concentrations in lactated Ringer solution as measured on our analyzer.](http://ajpregu.physiology.org/)
**DISCUSSION**

Multiple studies have shown that the intramembranous absorption of amniotic fluid across the amnion and into the underlying fetal vasculature plays a primary role in regulating amniotic fluid volume under diverse experimental conditions, including during intra-amniotic fluid infusions (5, 8, 10, 11, 13, 14, 17). The present study provides new insights into the mediators of the regulation of intramembranous absorption by showing that the increase in intramembranous absorption during intra-amniotic fluid infusion is primarily due to dilution of a nonrenal, nonpulmonary inhibitor(s) present in the amniotic fluid. This conclusion is based on four observations in the fetal sheep model: 1) regulation of intramembranous absorption rate occurs by either fetal membrane stretch and/or chemicals in the amniotic fluid (2), 2) membrane stretch does not alter amniotic fluid volume (7), 3) there is an absence of inhibitors or stimulators in fetal lung liquid secretions (9, 12, 13), and 4) the present results showing that the increase in the rate of intramembranous absorption in response to intra-amniotic infusion occurred even in the absence of the renal intramembranous stimulator.

The present study also suggests that the increase in intramembranous absorption rate during intra-amniotic infusion is not due to changes in amniotic fluid and/or fetal blood concentrations of the major solutes, consistent with previous studies (1, 9, 18). This conclusion is based on the observations that the intramembranous concentration differences for potassium, sodium, calcium, chloride, and glucose were unchanged during the infusions. Further, although amniotic lactate concentration increased during the infusions, that would not explain the elevation in intramembranous absorption rate during the infusions because any potential osmotic effects of the elevated amniotic lactate would tend to reduce rather than increase absorption rate. Experimentally, elevated amniotic lactate concentrations do not alter amniotic fluid volume (16). Finally, physiological changes in amniotic osmolality and composition produce relatively small changes in intramembranous absorption rate (6). Thus, through the process of elimination, the present findings are consistent with the presence of a nonrenal, nonpulmonary inhibitor of intramembranous absorption in the amniotic fluid that was diluted by intra-amniotic fluid infusions. The potential inhibitor appears to be either of fetal membrane origin or perhaps mucoid secretions from the fetal head or both. Further, this regulator of intramembranous absorption rate appears to alter only the active, unidirectional component of intramembranous absorption (2, 4, 9).

It is possible that alterations in the composition of the fetal blood that perfuses the intramembranous pathway could have regulatory effects. This could occur during hypoxia because fetal hypoxia produced by embolization of the fetal side of the placenta (8) or by lowering maternal inspired oxygen content (17) is associated with increased intramembranous absorption without an increase in amniotic fluid volume. However, the fetuses in the present study were not hypoxic. Alternatively, the increase in intramembranous absorption during fetal hypoxia may be mediated by changes in fetal urine composition and/or flow rate, as large increases in fetal urine production occur during sustained fetal hypoxia (17). The relative contribution of increased renal production vs. dilution of nonrenal amniotic fluid inhibitors to the rise in intramembranous absorption during fetal hypoxia has yet to be explored.

Our recent study demonstrated the presence of a renal stimulator of intramembranous absorption based on the observation that fetal urine replacement resulted in a large decrease in intramembranous absorption rate that was independent of the major solute concentrations and osmolality of the replacement fluid (2). However, it is not known whether this renal stimulator is produced at a constant rate or perhaps at increasing rates as urine flow increases. In the present study under basal conditions, intramembranous absorption rate and urine flow rate were not significantly correlated, suggesting that the renal stimulator may be produced at a constant rate and, thus, would be diluted when urine production increased and further diluted upon entry into the amniotic fluid. Although there was a significant upward shift in the relationship between urine flow and intramembranous absorption rates during the infusion when urine entered the amniotic fluid (Fig. 3, top), they remained nonsignificantly related, suggesting that the renal excretion of the intramembranous stimulator changed minimally with alterations in urine flow rate.

Unexpectedly, urine replacement during intra-amniotic fluid infusion did not reduce the intramembranous absorption rate compared with intra-amniotic infusion without urine replacement. If produced at a constant rate, the urinary concentration of the stimulator would be reduced during the infusions because urine flow rate increased and would be further diluted by the infused fluids, thereby reducing its amniotic concentration and, thus, its effectiveness. Another possibility is that the renal stimulator may be weak compared with the effects of dilution of the inhibitors that are present in amniotic fluid. This is consistent with the observation that intramembranous absorption rate remained low following fetal esophageal ligation while urine continued to enter the amniotic space, resulting in high amniotic fluid volumes (12). Although high amniotic fluid volumes comparable to those following esophageal ligation occurred following intra-amniotic fluid infusion in this and other studies (5, 10, 13), an important difference is that large increases in intramembranous absorption rate occur during intra-amniotic fluid infusion, whereas absorption rate remained low following esophageal ligation. These observations suggest that the inhibitor present in amniotic fluid is not only powerful but also is removed primarily by fetal swallowing.

One important observation that provides insight into the effectiveness of the amniotic fluid inhibitor(s) is that the fetuses with the lowest control urine flow rates underwent the greatest increases in intramembranous absorption rate during the infusions, whereas fetuses with the highest urine flow rates underwent the smallest infusion induced increases in absorption rate (Fig. 3, bottom). This suggests that, under basal conditions, the amniotic fluid inhibitor is continually being diluted by the entry of fetal urine into the amniotic fluid.

Collectively, competing inhibitor(s) and stimulator(s) of intramembranous absorption appear to be present in amniotic fluid, so it may be difficult to predict the changes in intramembranous absorption that occur under experimental conditions. For example, during a 3-day, 3 l/day washout of amniotic fluid in late-gestation sheep, amniotic fluid volume almost doubled (18). While intramembranous absorption rate was not measured, the increase in volume suggests a decrease. An increase in volume, coupled with a reduction in absorption rate, sug-
suggests that, under control conditions, the amniotic fluid stimulator(s) appears to be dominant over the inhibitor(s) of intramembranous absorption. In contrast, during fluid infusion, the increase in volume coupled with an increase in absorption suggests that the amniotic fluid inhibitors appear to dominate. Why the difference is unclear, but it may be related to the more extensive volume changes in the present study.

Previous studies from our laboratories have suggested that intramembranous absorption rate does not appear to depend on amniotic fluid composition (1, 3). This conclusion contrasts with other (18), more recent (2) studies, as well as the present studies, demonstrating the presence of stimulator(s) and inhibitor(s) of intramembranous absorption in amniotic fluid. The likely explanation of this apparent conflict is that, in the previous studies, the experimental condition may have equally altered the effects of the inhibitors and stimulators, with the result that little change in absorption rate occurred whereas, in the recent and present studies, the effects of the regulatory factors were experimentally isolated from each other, with the result that their presence could be demonstrated.

**Perspectives and Significance**

It has been clear for more than a decade that a comprehensive understanding of the regulation of amniotic fluid volume requires deciphering the mechanisms that regulate intramembranous absorption rate (15). Although significant progress has been made, achieving this goal has been slow not only because of the complexities of the multiple amniotic inflow and outflow pathways but also because intramembranous absorption rate cannot be measured directly but instead must be calculated from other measured flows and amniotic fluid volume changes. For the first time, the complex pieces of the regulatory puzzle are starting to come together. Through the process of elimination, this study reveals the existence of an important regulator of intramembranous absorption, i.e., amniotic fluid inhibitor(s). This type of inhibitor is nonrenal and nonpulmonary in origin. The present finding complements our recent demonstration of a fetal urine-derived stimulator of intramembranous absorption (2). Future challenges are the identification of the inhibitors and stimulators and their sources, as well as unraveling their interactions that regulate the passive and active components of intramembranous transport that ultimately determine intramembranous absorption rate and, thus, amniotic fluid volume.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


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