Absorption of amniotic fluid by amniochorion in sheep

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METHODS

Surgical preparation. The surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC). Ten time-bred ewes carrying singleton lambs were fasted for 24 h with access to drinking water and were operated on at 120 ± 1.5 (SD) days of gestation. Anesthesia was induced with an intravenous dose of 400 mg ketamine and 10 mg diazepam. The ewes were then intubated, and anesthesia was continued with a 50:50 mixture of oxygen and nitrous oxide and about 1% halothane. The concentration of halothane was adjusted to ensure a surgical level of anesthesia in both the ewe and the fetal lamb.

Figure 1 summarizes the experimental preparation. After opening the maternal abdomen and the uterus, we attached the amniochorion to the inside of the uterus with interrupted sutures. We inserted indwelling arterial and venous catheters for fetal blood sampling and pressure measurements. The gastric end of the cut esophagus was connected to the pulmonary end of the cut trachea by means of a short piece of vinyl tubing. This was done to eliminate both the inflow of lung fluid into the amnion and the drainage of amniotic fluid by swallowing. The distal ends of the esophagus and the trachea were ligated. An indwelling catheter was advanced into the urinary bladder via the urachus, and the allantoic end of the urachus was ligated. The ligation eliminated all known fluid inflow into the allantoic sac, and the vesicular catheter permitted the later drainage of fetal urine. Two or three whiffle balls, each attached to a large-bore catheter, were attached to the skin at the neck, back, groin, or hind leg.

It was proposed therefore that amniochorionic absorption is homeostatic in the service of volume control (9).

Although the site of amniochorionic absorption in sheep is believed to be the microvascular bed of the chorion (5), the question how this amniochorionic absorption is effected remains unanswered. Amniotic fluid absorption is often stated to be osmotic, because amniotic fluid is generally somewhat hypopsmotic with respect to fetal plasma, but direct evidence seems to be lacking. It is also possible that absorption depends on the difference in colloid osmotic pressures between amniotic fluid and fetal plasma. In the present experiments we investigated these possibilities by measuring amniochorionic absorption in the presence of induced changes in the osmolar and oncotic gradients between amniotic fluid and fetal plasma.

IN THE SHEEP, the inflows of fluid into the amnion are fetal urination and fetal lung fluid production, and the outflows are fetal swallowing and fluid absorption into the vasculature of the amniochorion (4). Direct exchange between amniotic fluid and maternal plasma is quantitatively insignificant (1, 2, 4). Experimentally induced changes in amniotic fluid volume in sheep are not followed by appropriate changes in lung fluid production (7) nor in urine production (9). It follows that, at least under such experimental conditions, neither lung fluid production nor urination serves a regulatory role in the control of amniotic fluid volume. Whether fetal swallowing serves a regulatory role remains possible but inconclusive (15). There is evidence, however, that amniochorionic absorption in sheep increases appropriately when swallowing is not possible (13) and also when the amniotic fluid inflow is experimentally augmented by amniotic infusion of Ringer solution (9).
of the fetus. These hollow balls with multiple side holes permitted the drainage of amniotic fluid without obstruction of the catheter ends by loose folds of amniotic membrane (2). Three small-diameter vinyl catheters were sewn to the fetal skin near the whiffle ball sites for sampling of amniotic fluid and for intra-amniotic infusion of fluids. The uterus and attached amniochorion were then sewn shut with no more than one catheter emerging between each pair of interrupted sutures. The closed uterine incision was oversewn with a continuous suture. No antibiotics were used with the exception of a single postsurgical dose of 10⁶ units of penicillin-G into the amnion as prophylaxis against Clostridia. Aerobic and anaerobic cultures were taken afterward to confirm the sterility of the preparations.

The animals recovered in a private pen for a minimum of 3 days (average 4.8) before any experiments were begun. After completion of the experiments, the animals were killed with an intravenous injection of a commercial euthanasia solution, as approved by the IACUC.

**Experimental protocols.** The experiments began with a measured volume of amniotic fluid of known osmolality. The subsequent inflow of fluid into the amnion was monitored or controlled. The sum of the initial and inflow volumes constituted the volume of fluid to be accounted for at the end of each experiment. Thus the volume of fluid retrieved at the end of the experiment showed the volume of fluid that had been absorbed (or produced) by the amniochorion in the course of the experiment.

After the ewe had been placed in a stanchion in the laboratory, the volume of amniotic fluid was measured and sampled by draining it into sterile flasks by a modification of the technique of Dickson et al. (8). Because of the dead space of the whiffle balls (70–100 ml total, depending on their number), some fluid inevitably stayed behind after drainage of the amniotic fluid. These volumes and their solute contents were accounted for in all calculations. Depending on the experiment, the drained volume was either reinfused into the amnion or replaced with a volume of mock amniotic fluid of the desired composition and osmolality.

From this time on, all fetal urine was continuously drained into a sterile flask for the purpose of measuring fetal urine production. A roller pump either reinfused the drained urine into the amnion or infused exogenous mock amniotic fluid at the same rate that urine was produced while the urine was discarded; the latter procedure did not disturb the normal fluid content of the conceptus. The Gilson Minipuls-3 infusion pump (72 rue Gambetta, BP45, Gilson Medical Electronics; Villiers-le-Bel, France) was operated by a relay that held the urine in the flask at a constant level (Fig. 1). When exogenous fluid was infused instead of urine, a second line in the roller head of the pump assured a flow rate that was the same as that of the production of urine to within ± 2%. After the amniotic fluid drained at the onset of the experiment had been reinfused or replaced with fluid of the desired composition and the urine or replacement infusion had been started, samples of fetal blood and amniotic fluid were taken daily or more frequently. At the end of the experimental period, we again took samples. The amniotic fluid was drained as described or collected at autopsy and analyzed. If more than one experiment was performed on the same conceptus, the next experiment was started at the time the preceding experiment was terminated by replacing the drained fluid with a fluid of another composition.

In three fetuses, ¹²⁵I-labeled human serum albumin was injected intraveously to investigate the transfer of this macromolecule from the intravascular space into the amnion of these surgically modified preparations.

**Instrumentation.** Pressures were measured by means of Abbott Transpac-IV transducers and a Gould CL800232 polygraph or an InstruNet system, operated by GW Superscope II software installed in a Macintosh G3 computer. Before each experiment, the transducers were calibrated against a mercury manometer. Vascular pressures were referred to amniotic fluid pressure. Arterial blood oxygen saturations, contents, pH, and gas pressures were measured in an Instrumentation Laboratories 842 CO-Oximeter and an Instrumentation Laboratories 1610 pH/Blood Gas Analyzer. Osmolalities were determined by freezing point depression in an Advanced model 3300 Micro Osmometer. Activities of ¹²⁵I were obtained with a Wallac 1470 Wizard gamma spectrometer.

**Solutions.** Solutions of various concentrations (see RESULTS AND DISCUSSION) of NaCl (Mallinkrodt) and mannitol (Sigma) were made with distilled water and sterilized in an autoclave. Plasma albumin solutions were prepared by dissolving lyophilized bovine serum albumin (Sigma A7906) into NaCl or mannitol solutions and were sterilized by filtration through sterile Corning no. 430770 0.45 micrometer cellulose acetate membranes.

**Analytic methods.** Total protein concentrations were determined by means of a commercial version of the Lowry method (Sigma Diagnostics). ¹²⁵I-labeled albumin concentrations were determined after the removal of small concentrations of loose label by precipitation in equal volumes of 10% trichloracetic acid.

**Data presentation and statistical methods.** Data are given as means ± SE, unless otherwise noted. Statistical calculations were performed by the GraphPad Prism version 3.02 for
RESULTS AND DISCUSSION

Condition of the animals. Twenty-one experiments were performed on 10 fetuses. The average duration of the experiments was 66 ± 5 h (2.7 days). Table 1 shows the fetal control data before each of the 21 experiments were begun. The control data before the first experiment by paired t-tests, and no statistically significant changes were found in any of the parameters listed in Table 1, indicating that the experiments did not affect the health of the fetuses. Mean autopsy weight of the fetuses was 3.84 ± 0.37 kg at a gestational age of 132 ± 2 days, which is normal, and no fetal abnormalities, other than those surgically created, were found at postmortem examination.

Crystalloid osmolality and amniochorionic absorption. In five control experiments, the drained amniotic fluid was reinused, and the drained urine was infused also. The osmolality differences between amniotic fluid and fetal plasma in these experiments were recorded but not experimentally controlled. In these experiments, mean fluid absorption was 21.9 ± 3.6 ml/h. This is in the range of previously recorded flow rates, as summarized in Ref. 9, indicating that the minor surgical modifications of our original preparation had no effect on amniochorionic absorption. These data are shown as squares in Fig. 2.

In five experiments, the osmolalities of the amniotic fluids were changed by infusing NaCl solutions of various concentrations. In these experiments, as in the urine infusion experiments, the osmolality of the amniotic fluid depended mostly on electrolyte concentrations. The differences between the amniotic fluid and the fetal plasma osmolalities are shown as open circles in Fig. 2. In three experiments the osmolality of the amniotic fluid was changed by replacing drained amniotic fluid and drained urine with solutions of mannitol. These data are shown as open triangles in Fig. 2.

The crystalloid (electrolytes and mannitol) osmolalities of the amniotic fluid strongly affected the rate of fluid absorption (or production) by the amniochorion, and this relation was statistically highly significant (see Oncotic pressure and amniochorionic absorption). Although this result confirmed the expectation that amniotic fluid absorption or production was affected by the osmolar gradient, Fig. 2 also shows a positive intercept, suggesting that absorption was still taking place in the absence of a difference in osmolality and that, therefore, there might exist an additional absorptive process that was independent of crystalloid osmotic pressure.

Oncotic pressure and amniochorionic absorption. To determine whether the rate of fluid absorption was affected by onocytic pressure differences between amniotic fluid and fetal plasma, we infused bovine albumin solutions into the amnion in eight experiments. In seven of the experiments, the albumin was dissolved in NaCl solutions of various osmolalities and one in a mannitol solution. In these experiments, the onocytic gradient that normally strongly favors fetal plasma was reversed, the mean protein concentration being +14.1 ± 3.4 g/l (P < 0.005), higher in the amniotic fluid than in the plasma. In contrast, the mean difference in the protein concentrations of the amniotic fluids and the fetal plasmas in the 13 experiments in which no protein was infused into the amnion was -29.0 ± 2.6 g/l (P < 0.001). The data points of the experiments in which protein was infused into the amnion are shown as filled symbols in Fig. 2.

We performed a multiple linear least squares regression with fluid absorption as the dependent variable as a function of the differences in osmolalities and the differences in protein concentrations. The zero-zero intercept was a fluid absorption of 24.9 ± 5.6 ml/h (P <
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0.001), and the regression coefficients were \(-0.66 \pm 0.12 \text{ ml/h} \) per mosmol/kg-osmolality difference \((P < 0.001)\) and \(+0.07 \pm 0.20 \text{ (not significant) ml/h} \) per g/l-protein concentration difference, with \(r^2 = 0.63, P < 0.0003\). Clearly, there was a highly significant association between fluid absorption and osmolality difference but no demonstrable oncotic effect.

For this reason, a bivariate least squares linear regression was performed on all 21 data points, relating fluid absorption to the differences in osmolality. This regression is shown in Fig. 2. The zero osmolality intercept was 23.83 \pm 4.71 ml/h \((P < 0.001)\), and the regression equation was \(J_{\text{AF}} = +23.8 - 0.65(\text{Osm}_{\text{AF}} - \text{Osm}_{\text{FP}}) \text{ ml/h} \) \((P < 0.0001)\), where \(J_{\text{AF}}\) is the chorioamniotic absorption rate of amniotic fluid (in ml/h) and \(\text{Osm}_{\text{AF}}\) and \(\text{Osm}_{\text{FP}}\) are the osmolalities of amniotic fluid and fetal plasma (in mosmol/kg), respectively.

**Effect of amniotic fluid osmolality on fetal plasma osmolality.** Figure 2 shows that the amniotic fluid osmolalities ranged from 53 mosmol/kg hyposmolar to 98 mosmol/kg hyperosmolar with respect to fetal plasmas of the same experiment. The actual range of amniotic fluid osmolalities was somewhat greater, being from 231 to 403 mosmol/kg because fetal plasma osmolalities shifted somewhat in the direction of amniotic fluid osmolality. However, the range of fetal plasma osmolalities in these experiments was very much smaller than the range of amniotic fluid osmolalities, remaining only between 280 and 305 mosmol/kg, because the fetal plasma osmolality is tightly coupled to the maternal plasma osmolality by equilibration across the placenta.

**Fate of infused protein.** Because the amounts of protein infused and recovered were known from the products of the concentrations and the volumes, the amounts of protein that were absorbed from the amniotic fluid could be calculated. This absorption rate was \(1.8 \pm 0.4 \text{ g/h} \) \((n = 8, P < 0.001)\). To confirm that the fetal circulation can accommodate the amounts of bovine albumin that were absorbed from the amniotic fluid, we infused a bovine albumin solution of 108 g/l in isotonic Ringer solution directly into the vein of one fetus before any other experiments were performed. After 30 g of protein had been infused in 21 h, fetal plasma protein concentration had risen only from 37 to 42 g/l, and fetal hematocrit had decreased from 35 to 31%. This further agrees with unpublished experiments performed in our laboratory in which homologous protein infused into sheep fetuses was rapidly cleared from the circulation.

**Fluid flows associated with protein absorption.** We considered it unlikely that the protein that was absorbed was carried by the fluid flows driven by the differences in crystalloid osmolalities, because such flows require the existence of nonzero reflection coefficients for the crystalloids \((6, 11)\). This would be possible only in passages too narrow to permit the passage of proteins. There was the additional consideration that protein concentration differences did not demonstrably affect the water absorption rate, indicating that the protein reflection coefficients in the passages that did allow protein absorption were close to zero. Thus the paths that allowed protein to pass did so without discrimination of molecular size. Because both the protein absorption rates and the protein concentrations were known, the flows of fluid associated with protein absorption rates could be calculated. The mean flow was \(33.8 \pm 6.1 \text{ ml/h} \) \((P < 0.001)\), a value that was not significantly different \((P = 0.2)\) from the intercept of 23.8 ml/h of the linear regression of water absorption as a function of the difference in osmolalities \((\text{Fig. 2})\).

Absence of diffusional transfer of albumin between plasma and amniotic fluid. In three fetuses, \(^{125}\text{I}\)-labeled human albumin was injected intravenously. Figure 3 shows the concentrations of trichloracetic acid-precipitable label in fetal plasma and amniotic fluid during the next 24 h and beyond. Although there was detectable radioactivity in the amniotic fluid near the ends of these experiments, the amounts were so small that they argued against a diffusional mechanism to account for the absorption of the bovine albumin from the amniotic fluids.

In conclusion, fluid absorption by the amniocorion was strongly affected by the difference in the osmolalities of amniotic fluid and fetal plasma, confirming the conventional belief that amniotic fluid absorption is driven by crystalloid osmotic pressures. The present experiments demonstrate, however, that there must be an additional path. Absorption continued in the presence of a zero osmolality difference, and there was significant absorption of plasma albumin by a process that was nondiffusional in nature. The volume flow through this additional path was far from negligible, being of the order of 25 ml/h, as judged from the intercept in Fig. 2 and the fluid flows calculated from the protein absorption rates.

The barrier that separates amniotic fluid from fetal plasma consists of a layer of amnion cells, extracellular matrix, and the endothelium of the chorial microvasculature \((5)\). The existence of osmotically driven water

![Fig. 3. Concentrations of \(^{125}\text{I}\)-labeled albumin in fetal plasma and amniotic fluid after a single intravenous dose at time 0 in three fetal lambs. Data normalized by setting plasma concentrations at 19 h equal to 100 and converting other values accordingly.](http://ajpheart.org)
and crystalloid flows across these layers presents no new theoretical challenges (6). We had anticipated (9) that, in addition to the flow across the capillary walls of the amniochorionic microcirculation that was driven by crystalloid osmotic pressure, there would be a second flow driven by a simple Starling-type hydrostatic-colloid osmotic mechanism. However, the present experiments clearly refuted this. A recent modification of the capillary Starling mechanism on the basis of a more detailed structural model of the capillary barrier (10) does not affect this conclusion.

Thus the anatomic nature of the path that allows the absorption of additional fluid and protein is not known. This path has the following physiological properties: 1) it absorbs fluid against combined osmotic and oncotic gradients; 2) it absorbs fluid against a hydrostatic gradient because the pressure in the amniotic fluid is lower than the pressure anywhere in the fetal circulation, including the atra (14); and 3) it allows the passage of plasma albumin by a process that is nondifusional and unidirectional.

**Perspective.** The physiological properties of the additional path of amniotic fluid absorption closely mimic those of the lymphatic system. However, a lymphatic system in the amniochorion has not been described, and the umbilical cord is known not to contain lymphatic trunks (3, 12). Although the force of this argument is diminished by the consideration that conventional methods for demonstrating terminal lymphatics are not likely to work in the amniochorion, the anatomic basis for this path remains obscure.

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**REFERENCES**