Pharmacokinetics of Protein C and Antithrombin in the Fetal Lamb: A Model to Predict Human Neonatal Replacement Dosing

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Key Words

Protein C · Antithrombin · Pharmacokinetics · Lamb · Replacement protein

Abstract

Background: The preterm infant is at risk for consumptive coagulopathy and thrombosis due to late maturation of coagulation regulatory proteins. Replacement proteins are available, but neonatal pharmacokinetic data are lacking.

Objective: The objective was to determine the pharmacokinetic properties of antithrombin (AT) and protein C (PC) in order to provide data for estimating doses in human infants.

Methods: A catheterized ovine model was used to determine pharmacokinetic properties of AT and PC, including plasma recovery, volume of distribution (V_d), clearance (Cl) and half-life ($t_{1/2}$), in the fetal lamb relative to the ewe. **Results:** AT studies showed statistically significant differences between ewes and fetuses in recovery ($p < 0.0001$), V_d ($p = 0.0002$) and Cl ($p < 0.0001$). The AT $t_{1/2}$ was significantly shortened among fetuses (5.55 h, 95% CI: 4.01–7.08) compared to ewes (18.7 h, 95% CI: 11.6–25.8). PC recovery ($p < 0.0001$), V_d ($p < 0.0001$) and Cl ($p = 0.004$) differed significantly between ewes and singleton fetuses as did the $t_{1/2}$: 3.86 h (95% CI: 3.35–4.36) and 11.9 h (95% CI: 10.9–12.9) in the singletons and ewes, respectively. All PC parameters were significantly different for twins compared to ewes. **Conclusions:** AT and PC

show decreased recovery and $t_{1/2}$ in the fetal lamb. These data can be used to estimate dosing for human neonates in comparison with human adult dosing recommendations.

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Introduction

Newborn infants, particularly sick preterm neonates, are at increased risk of disseminated intravascular coagulation (DIC) and catheter-related thrombosis [1, 2]. In part, the increased rate of these prothrombotic syndromes during the perinatal period is attributed to delayed fetal and neonatal physiological maturation of the coagulation-regulatory proteins antithrombin (AT) and protein C (PC) [3, 4]. Further reductions in plasma levels of PC and AT have been determined in sick infants [5, 6]. Deficiencies in coagulation-regulatory proteins are compounded in neonates with thrombosis because standard anticoagulation using unfractionated and low-molecular-weight heparin is often difficult to achieve in newborn infants secondary to very low levels of AT [7, 8].

Infants with severe genetic AT deficiency have developed life-threatening thrombosis during the perinatal period [9–11]. Acquired AT deficiency causes severe thrombosis in infants undergoing cardiopulmonary bypass or extracorporeal membrane oxygenation [12, 13]. A

knockout mouse model of AT deficiency shows embryonic lethality by 16.5 gestational days [14]. The plasma half-life ($t_{1/2}$) of AT has been reported at 59 h in stable AT-deficient adults [15]. This long $t_{1/2}$ supports the feasibility of intravenous protein replacement, based on clinical indications. Concentrates of human plasma-derived, highly purified, viral-inactivated AT are licensed and have been used to treat adults, children and neonates with genetic deficiencies around surgery, childbirth and episodes of thrombosis [9–11, 15–17]. In addition, AT concentrate has been given to infants and children with acquired deficiencies secondary to sepsis, cardiopulmonary bypass surgery, DIC, chemotherapy with L-asparaginase and liver transplantation [12, 17–21]. Human infants have a mean plasma concentration of AT of 50 U/dl at term birth and reach the adult level of 100 U/dl by 6 months [3, 4, 6, 22]. Stressed preterm infants have levels of AT as low as 10 U/ml [6, 23].

Infants with homozygous or compound heterozygous genetic deficiencies of PC present in the neonatal period with purpura fulminans, DIC and large-vessel thrombosis [24–26]. The $t_{1/2}$ of PC in plasma has been reported at 6–8 h [27, 28]. A concentrate of human-plasma-derived, highly purified, viral inactivated PC has been licensed in Europe and the USA [27–29]. PC concentrate has been used in patients with heterozygous genetic deficiencies around childbirth [27] and in infants and children with homozygous or compound heterozygous genetic deficiencies during episodes of purpura fulminans, DIC and large-vessel thrombosis as well as for the prophylaxis of these lesions [28, 29]. The mean plasma concentration of PC in healthy term infants is 40 U/dl and does not achieve adult levels until later childhood, and possibly puberty [3–6, 30]. Very low levels of PC, less than 10 U/dl, have been reported in sick preterm infants and appear to convey an increased risk of thrombosis [5, 6].

Replacement therapies may be beneficial in sick neonates with very low levels of AT and PC. However, little is known regarding the pharmacokinetics of these proteins in the preterm and term infant. Because available replacement concentrates of AT and PC are derived from pooled human plasma, it is not ethical to infuse them into stable human infants for the purposes of pharmacokinetic research. We chose a fetal ovine (lamb) model to compare plasma recovery and elimination following infusion of replacement concentrates of these proteins in comparison to ewes. Previous studies of coagulation proteins in fetal and adult sheep (ewes) in our lab have shown good comparability with human (preterm and term neonate, child, adult) data [31–33].

Methods

Preparation of the Animal Model

Pregnant Columbia-Rambouillet sheep with known gestational age were operated on between 70 and 80 days of gestation to place maternal arterial sampling and venous infusion catheters or at approximately 120 days of gestation for placement of both maternal and fetal arterial sampling catheters and venous infusion catheters. At both times of gestation, surgery was performed using either of two anesthesia protocols: intravenous pentobarbital sedation (15 mg/kg) and spinal anesthesia (tetracaine HCl, 10 mg in hypertonic glucose) or intramuscular acetylpromazine (1 mg/kg) with intravenous ketamine anesthesia (12–15 mg/kg loading dose with 0.3–0.5 mg/kg/min continuous infusion). Catheters for infusion and sampling were placed into fetal and maternal vessels using standard surgical procedures as previously described [31]. Fetal catheters included an arterial catheter for sampling inserted via the dorsal pedal artery and advanced to the lower abdominal aorta and a venous catheter for infusion inserted via the dorsal pedal saphenous vein and advanced into the lower abdominal vena cava. Maternal arterial (sampling) and venous (infusion) catheters were inserted into the maternal femoral artery and vein via a groin incision. All catheters were tunneled subcutaneously and kept within a plastic pouch attached to the maternal flank. The catheters were flushed daily to every other day with 0.9% wt/vol NaCl in H₂O that contained 30 U/ml sodium heparin. After surgery, the sheep were allowed to recover at least 4 days before study. All protocols were approved by the Animal Care and Use Committee of the University of Colorado Denver, which is accredited for laboratory animal use by the National Institutes of Health, the United States Department of Agriculture, and the American Association of Laboratory Animal Care.

Laboratory Methods

Plasma from healthy adult sheep was used to isolate ovine PC as previously described [32]. Ovine PC was used to raise polyclonal rabbit anti-ovine PC antibodies. An assay for ovine plasma PC concentration was established using the Laurell rocket technique as previously described [32]. Ovine plasma AT activity was determined using a chromogenic assay as previously described [31]. A Laurell immunoassay for human PC in ovine plasma was established using a polyclonal rabbit anti-human PC antibody. This assay did not detect ovine PC in a plasma pool prepared from 26 healthy adult male and female sheep. A similar assay for human AT in ovine plasma was established using a polyclonal rabbit anti-human AT antibody. This assay also failed to detect ovine AT in the ovine plasma pool described above.

Blood samples were collected from the ewe and her fetus on one or more occasions. The catheters were first cleared by removing 3 ml of blood, and then 0.9-ml blood samples were drawn into 0.1 ml of 3.8% sodium citrate anticoagulant. The samples were centrifuged at 1,800 g for 20 min at 4°C. Aliquots of plasma were subsequently frozen at –70°C until the time of assay.

Establishment of Normal Adult and Gestational Ovine Plasma PC Concentrations

Separate aliquots of frozen plasma were thawed for assay of PC and AT. A standard ovine plasma pool was prepared from 26 healthy nonpregnant adult sheep. Normal adult values for ovine PC and AT were derived from these individual plasma samples,

Table 1. Pharmacokinetic calculations of AT and PC infusions

	$t_{1/2}$, h	k, h ⁻¹	Cp ₀ , U/dl	Recovery U/dl per U/kg	V _d , dl/kg	Cl, dl/kg/h
<i>Antithrombin</i>						
Ewes (n = 3)						
Mean	18.7	0.04	51.9	2.24	0.49	0.02
SD			5.37	0.15	0.05	0.003
95% CI	11.6–25.8	0.02–0.05				
Fetuses (n = 4)						
Mean	5.55	0.13	61.6	0.93	1.14	0.14
SD			5.26	0.13	0.10	0.01
95% CI	4.01–7.08	0.09–0.16				
p ¹				<0.0001	0.0002	<0.0001
<i>Protein C</i>						
Ewes (n = 6)						
Mean	11.9	0.06	89.6	3.08	0.33	0.02
SD			20.8	0.28	0.03	0.003
95% CI	10.9–12.9	0.05–0.06				
Singleton fetuses (n = 4)						
Mean	3.86	0.18	113.1	1.62	0.62	0.12
SD			10.6	0.15	0.06	0.02
95% CI	3.35–4.36	0.16–0.20				
p ¹				<0.0001	<0.0001	0.004
Twin fetuses (n = 6)						
Mean	5.56	0.12	78.7	1.30	0.81	0.11
SD			2.82	0.26	0.16	0.04
95% CI	5.09–6.03	0.11–0.14				
p ¹				0.0008	0.002	0.003

¹ p for the comparison between ewes and fetuses.

with the pool mean expressed as 1 U/ml as previously described [33]. Study samples were read against curves constructed using dilutions of the normal ovine pool.

Conduct of Recovery and Half-Life Studies of PC and AT in the Ewe and Fetal Lamb

AT or PC was infused with a syringe pump over 10 min. Blood samples were removed at various times following the end of the infusion. Baseline samples were collected in duplicate at least 10 min apart and assayed for both ovine and human PC antigen or human AT antigen and total AT activity. Assays were performed in duplicate and reported as mean results.

Dosing for the ewe and the lamb was based upon pilot data and calculated to achieve similar peak plasma concentrations in the adult and fetus for comparison of plasma elimination curves. On different days, human plasma-derived AT concentrate (generously donated by the American Red Cross, Holland Laboratories, Silver Springs, Md., USA) was infused at a standard dose of 25 U/kg into the maternal ewe or 70 U/kg into her fetus. On separate occasions, human plasma-derived PC concentrate (generously donated by Baxter-Immuno, Vienna, Austria) was infused at doses of 25, 30 or 50 U/kg into the maternal ewe or 70 or 71 U/kg into her fetus. If the ewe carried a twin gestation, each fetus was stud-

ied on a separate day. Fetal weight was estimated from an intra-uterine fetal growth chart that has been established in our research facility for the Columbia-Rambouillet ovine breed. This chart accounts for different fetal growth rates in singletons and twins.

Data Analysis

SAS 9.1.3 was used for all analyses. The fetal plasma volume was adjusted to account for the dilutional effect of the placental plasma volume based on a study in which red blood cells and plasma were separately labeled to determine the plasma volume of the fetus alone and the plasma volume of the fetoplacental unit [34]. Creasy et al. [34] used ovine fetuses of the same gestational age as the sheep in our study and reported that plasma volume was 72.4 ml/kg for the fetus alone and 89.9 ml/kg for the fetoplacental unit. Thus, all fetal AT and PC levels were increased by a factor of 1.24.

A nonlinear mixed-effects model for one-compartment exponential decay was used to determine the $t_{1/2}$ of each protein in the maternal and fetal models separately. This model allowed us to account for unbalanced numbers of repeated measures, as well as the variability within and between sheep. Specifically, the model incorporated parameters for the random error around each mea-

Fig. 1. Elimination of human AT in ewes and fetal lambs. Each solid line represents the plasma concentration of AT for a ewe that was administered a dose of 25 U/kg. Each dashed line represents the plasma concentration of AT for a singleton fetus that was administered a dose of 70 U/kg. The inset graph uses the same scheme to show the natural logarithm of the plasma concentration of AT.

sured protein level, the random error around the elimination rate constant, and normal errors. Initial results indicated that the variance of the random error of the elimination rate constant was nearly zero, and the model fit the data better when this parameter was excluded. Half-lives and the rate elimination constant (k) are reported in hours and hours^{-1} , respectively, with a 95% CI. Log-linear plots for PC and AT were constructed to illustrate that the data are consistent with a one-compartment model.

The other pharmacokinetic parameters were calculated using standard formulae [35]. The mean and standard deviation (SD) were calculated for each parameter and reported separately for ewes, singleton fetuses and twin fetuses. The standard deviations for the twins were adjusted for the lack of independence between siblings using a repeated-measures analysis of variance. The p values for the comparisons between ewes and twins were similarly adjusted. Recovery is reported as units per deciliter assayed per units per kilogram infused. Volume of distribution (V_d) and clearance (Cl) are reported as deciliters per kilogram and deciliters per kilogram per hour, respectively. A p value of <0.05 was considered statistically significant. Singleton and twin gestations were analyzed separately for all calculations.

Results

Three ewes and 4 singleton fetuses were infused with AT, while PC was administered to 6 ewes, 4 singleton fetuses and 3 pairs of twin fetuses. The pharmacokinetic properties of human AT and PC were statistically significantly different in the ewe compared with the fetus (table 1). In order to confirm that the distribution of the proteins was confined to the animal to which the infusion

was administered, we sampled the fetus following maternal infusions and sampled the ewe following fetal infusions. We were unable to detect transfer of the proteins from ewe to fetus or from fetus to ewe.

The recovery of both PC and AT among ewes was approximately double that of singleton fetuses ($p < 0.0001$ for both infusates). As expected, the V_d in ewes was roughly half of the V_d in singleton fetuses (AT: $p < 0.0001$, PC: $p = 0.0002$). The Cl was markedly lower in the ewes compared to the fetuses (AT: $p < 0.0001$, PC: $p = 0.004$). Differences between ewes and fetuses in the AT and PC concentrations 15 min following the infusion (C_{p0}) were not tested, because these values are dose dependent, and doses were inconsistent across animals. The fetuses required a weight-specific dose ranging from 2 to nearly 3 times that of the ewe in order to achieve a similar plasma concentration 15 min following the end of the infusion.

AT distribution and elimination curves for each ewe and fetus are shown in figure 1. The $t_{1/2}$ of AT in the ewes was 18.7 h (95% CI: 11.6–25.8); among the fetuses, the $t_{1/2}$ was 5.5 h (95% CI: 4.01–7.08). PC distribution and elimination curves for each ewe and fetus are shown in figure 2. Although the curves for PC differ from those for AT and suggest different patterns of distribution and elimination, the overall results are similar. Among the ewes, the average recovery was 3.1 U/dl per U/kg (range: 2.6–3.3), while it was only 1.4 U/dl per U/kg for the fetuses (range: 1.1–1.8). The $t_{1/2}$ of PC also was statistically sig-

Fig. 2. Elimination of human PC in ewes and fetal lambs. Each solid line represents the plasma concentration of PC for a ewe. The open circles and open triangles indicate doses of 30 and 50 U/kg, respectively. All other ewes received a dose of 25 U/kg. The black and grey dashed lines represent singleton and twin fetuses, respectively. The closed circles indicate a dose of 50 U/kg; all other fetuses were administered a dose of 70 or 71 U/kg. The inset graph uses the same scheme to show the natural logarithm of the plasma concentration of PC. Doses are not depicted on the inset graph.

nificantly longer in the ewes (11.9 h; 95% CI: 10.9–12.9) than in both singleton and twin fetuses. In addition, the $t_{1/2}$ of PC was significantly shorter in the singleton fetuses (3.86 h; 95% CI: 3.35–4.36) compared with the twin fetuses (5.56 h; 95% CI: 5.09–6.03).

Discussion

We employed a chronically catheterized ovine model to study the pharmacokinetics of the coagulation regulatory proteins AT and PC to better understand neonatal deficiency states and to plan data-driven preventive and therapeutic strategies. We previously reported that ovine PC and AT can be detected in plasma of fetal lambs at 70 days of gestation (term gestation is 147 days) [33]. In that study, AT plasma concentrations increased linearly with gestational age. In contrast, PC plasma concentration did not increase from midgestation to the neonatal period and decreased slightly during the second and third trimester of gestation. Further experiments suggested that plasma concentration of PC in the ovine fetus is not regulated at the level of gene transcription.

In the current study, plasma recovery and $t_{1/2}$ of human AT and PC in the fetal lamb were approximately one-third to one-half of the corresponding values in the ewe. These data are similar to plasma $t_{1/2}$ studies of ovine fibrinogen conducted in neonatal lambs in comparison to

adult sheep [36]. Our data support both increased dose and dose frequency for replacement of AT or PC newborn infants relative to dosing in adults.

Very few data exist regarding pharmacokinetics of coagulation PC concentrates in newborn infants. Two reports of neonatal treatment of genetic PC deficiency with human plasma-derived PC concentrates describe an initial $t_{1/2}$ of 2–3 h, similar to results found in our fetal-maternal ovine model [37, 38]. In both of these reports, apparent PC $t_{1/2}$ extended to 10 h with continued treatment. Our studies did not include repeated dosing to determine whether ovine plasma elimination would be decreased with saturation of a possible reservoir compartment. Interestingly, 2 recent case reports of newborn infants with hemophilia B infused with recombinant factor IX have described extraordinarily high dosing requirements to maintain adult hemostatic plasma factor IX concentrations [39, 40]. In these 2 cases, factor IX requirements decreased over the first 2–4 months of life despite absence of an inhibitory antibody to factor IX. Factor IX is like PC in that it is also a vitamin-K-dependent coagulation protein with a similar molecular weight and volume of distribution, and it is possible that the two proteins share developmental pharmacokinetics. In contrast, infused factor VIII remains limited to the plasma compartment and has been reported to have a $t_{1/2}$ only modestly reduced from 12 h in healthy adults to 6 and 8 h in 2 preterm infants with hemophilia A [41].

It is evident that therapeutic armamentaria will continue to increase as more plasma protein replacements are available from recombinant and plasma-derived production. Consideration of replacement therapies for term and preterm infants with diagnosed coagulopathies will grow in parallel with expanded product availability. There exists a great need for pharmacokinetic data to guide coagulation protein replacement therapy in newborn infants. While the ovine model is limited in that data cannot be directly applied to human infants, ovine and other animal species can be employed to model developmental pharmacokinetics and to estimate comparative treatment dosing for human infants. At the same time, it is imperative to monitor plasma concentrations

around coagulation protein dosing in human infants in order to estimate crude pharmacokinetic data *ex vivo*. Initial treatment for a newborn may use a loading dose of AT or PC concentrate of 80–100 U/kg with resultant plasma activity determined by a rapid chromogenic assay at 1 h and 6–8 h following the infusion. The data would be used to calculate subsequent doses adequate to maintain a trough level of 30% or greater.

Acknowledgement

Supported by grants: National Institutes of Health, HL44586, and the American Diabetes Association (M.M.J.); and National Institutes of Health, DK35836 and HD20761 (W.W.H.).

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