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Original Research

Maternal-fetal Blood Major Crossmatching in Merino Sheep

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To determine the incidence of ex vivo incompatibility between ovine maternal RBCs and fetal plasma, we performed cross-matching of blood samples from ewes and from lambs delivered by cesarean section. Twenty-one date-mated singleton pregnant Merino ewes were anesthetized for cesarean delivery of the fetus. At the time of delivery, paired maternal and fetal blood samples were collected and subsequently separated for storage as packed red blood cells and fresh frozen plasma. Gel column major cross matching was performed within 2 wk. All fetus-dam crossmatches were major crossmatches, combining fetal (recipient) plasma with dam (donor) RBCs. 172 individual dam-dam cross matches were performed. Two of these tests were incompatible (1.2%). In addition, 19 fetal blood samples collected immediately after cesarean delivery were crossmatched with 21 maternal samples to generate 174 maternal-fetal individual cross matches. No maternal-fetal incompatibility reactions were observed. The results of this study demonstrate that all maternal donors and fetal recipients were compatible. In addition, the incidence of an incompatible crossmatch between adult ewes was 1.2%. These data suggest that lambs may not be born with antibodies against other blood types, but rather may acquire such antibodies at some time during early life. In addition, these data suggest the risk of incompatibility reactions between ewes of a similar breed and from a single farm of origin is very low.

Abbreviations: EVE, *ex vivo* uterine environment; RBCs, red blood cells

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Preliminary investigation of ex vivo incompatibility between ovine maternal red blood cells (RBCs) and fetal plasma was performed and reported an incidence of dam-dam and maternal (dam)-fetal incompatibility as 30.8% and 2.5% respectively.[7] The motivation for the preliminary investigation was the need for blood transfusions to preterm lambs housed in the context of an 'artificial placenta' or ex vivo uterine environment (EVE).[5,6,8,11] This EVE is a sheep model of an artificial version of extracorporeal support for a human fetus. This technology has continued to evolve and requires transfusions as whole blood, packed red blood cells (pRBCs) and plasma.[12] These needs warrant further investigations into maternal and fetal blood compatibility.

Given the complexity of the EVE model, a thorough understanding of the risk of potentially fatal transfusion reactions is prudent. Historical studies suggest that newborn lambs may not have antibodies against other blood types, nor immunogenic antigens on their RBC surface.[9] Data in lambs includes reference to R antigen, and anti-R antibodies, as well as O-antigen, and anti-O antibodies.[9] As preterm lamb fetuses do not ingest colostrum and are too young to have produced their own antibodies against foreign RBC antigens, they can potentially accept donor blood with minimal/negligible risk of an immunologic transfusion reaction. Investigations into the incidence of incompatibilities are required to determine the magnitude of this risk.

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The preliminary investigation had various limitations: a relatively small sample size; concern that the duration of storage of samples before cross matching may have overestimated the frequency of incompatibility reactions; and the collection of samples from only naïve fetuses, prior to any transfusions being administered.[7] The current study addresses these limitations, providing additional data to inform the decision-making process for administration of blood and blood products to preterm fetuses in the EVE model.

The purpose of this study was to investigate the incidence of incompatibility reactions in sheep, particularly between maternal and fetal units. Major cross matches were performed to focus on the incidence of incompatibility reactions between donor (dam) RBCs and recipient (fetal) plasma.

Materials and Methods

This study was approved by the Animal Ethics Committee at the University of Western Australia in accordance with the *Australian code of practice for the care and use of animals for scientific purposes*.[1]

Animals. Twenty-one date-mated singleton pregnant Merino ewes (*Ovis aries*) underwent anesthesia for cesarean delivery of the preterm fetus at 96 to 98 d of gestation. The ewes weighed 50.6 (±4.3) kg and were approximately 4 y of age. Ewes were sourced from a single commercial breeding farm in the southwest of Western Australia (Icon Agriculture, Darkan, Western Australia). Merino ewes were introduced to the farm prior to date mating to supply this project. For mating, the ratio of ram:ewe was 1:5 over a 24 h period. Estrus was induced in the

ewes with a controlled internal drug (progesterone) release device.

Prior to transport to the University farm, sheep were inspected by a veterinarian on the farm of origin to ensure the animals were healthy and in good body condition (body condition score 2.5 to 3.5 [max 5]). The ewes had been treated for intestinal parasites with abamectin, oxfendazole and levamisole twice during pregnancy, and had been vaccinated against Ovine Johne Disease (*Mycobacterium paratuberculosis*) as lambs. After arrival at the University farm, the ewes were monitored for body weight, body condition, food and water intake and general wellbeing. The ewes were introduced to the University farm paddocks at least 3 wk prior to the study. After one week in the paddock, the sheep were moved to a farm shed for one week and then into the animal facility one week prior to anesthesia and surgery. The ewes were initially fed a diet of pasture supplemented with oaten hay and approximately 40 g day pellets (Macco Feeds 707 pellets, Williams, Western Australia 6391). Once in the animal facility, sheep were fed a combination of chaff, oaten hay and pellets. Food was not withheld prior to anesthesia.

The ewes were anesthetized as previously described.[12] Briefly, acepromazine (0.01-0.03 mg/kg, ACP 2 injection, 2 mg/mL, Delvet Pty, Seven Hills, NSW, Australia) and buprenorphine (0.01 mg/kg, Temgesic 0.3 mg/mL, Reckitt Benckiser, Sydney, NSW, Australia) were administered by intramuscular injection, 40 min prior to induction of anesthesia. Anesthesia was induced with a combination of midazolam (0.25 mg/kg, Midazolam MYX, 5 mg/mL, Mayne Pharma International Pty, Salisbury South, South Australia) and ketamine (5 mg/kg, Ketamine Injection, 100 mg/mL, Ceva Delvet Pty, Asquith, NSW, Australia), delivered intravenously. The tracheas of the sheep were intubated and anesthesia was maintained with isoflurane (1.5% to 2.5%, IsoFlo, 1 mg/mL, Zoetis Australia, Rhodes, NSW, Australia) in oxygen, delivered through a circle breathing system. Mechanical ventilation was commenced immediately after induction of anesthesia to maintain normocapnia. The anesthetized ewes were monitored continuously during anesthesia; every 5 min the following were recorded: heart rate and rhythm, oxygen hemoglobin saturation, expired carbon dioxide, nasopharyngeal temperature, and oscillometric noninvasvie blood pressure (Surgivet V9203 multivariable monitor, Polymount GCX corporation, US.).

Blood collection. Whole blood was collected from the ewe (180 mL) to prime the artificial placenta circuit and aseptic surgery was performed to instrument the fetus for delivery to the EVE platform. After delivery of the fetus, 2 to 3 units (approximately 450 mL each) of whole blood were collected from the ewe into triple bag collection systems (Fresenius Kabi Compo-Flex Triple System, NSW, Australia). The ewe was then euthanized with intravenous pentobarbitone (160 mg/kg, Lethabarb 325 mg/mL, Virbac, N.S.W., Australia). Death of the ewe was confirmed by the absence of spontaneous ventilation, heartbeat, and corneal reflex.

During blood collection from the ewe, 2 mL aliquots of maternal blood were set aside for this study. After delivery of the fetus, blood was collected from an umbilical artery. During the period of support on the artificial placenta, any blood products administered to a preterm lamb were taken from a single maternal source. In a subset of animals, fetal blood was collected at the end of the EVE experiment, 5 d after delivery. Blood was put into tubes containing EDTA anticoagulant (BD vacuum phlebotomy tube, K2 EDTA, Sydney, Australia) and was separated by centrifugation within 2 h for storage of packed RBCs at 4 °C and plasma at -80 °C. At the end of the experiment, fetal lambs were euthanized with intravenous pentobarbitione (160 mg/kg, Lethabarb 325 mg/mL, Virbac, N.S.W., Australia).

Crossmatching. Gel column crossmatching was performed within 2 wk of sample collection. All fetus-dam crossmatches were considered major crossmatches, combining fetal (recipient) plasma with dam (donor) RBCs. In addition, allogenic dam-dam crossmatches were performed for comparison. Autologous negative controls (recipient plasma with its own RBCs) were also performed. All gel column crossmatches were performed according to the manufacturer's instructions ("Gel", BioRad ID-Cards, NaCl, Enzyme Test and Cold Agglutinins DiaMed GmbH, Cressier, Switzerland) as described previously.[7] Briefly, 10 uL of packed RBCs from each donor was added to a tube with 1 mL of low ionic strength solution (LISS, ID-Diluent 2, DiaMed), and mixed gently, to prepare a 1% RBC suspension. For each donor-recipient combination, 50 µL of the 1% donor RBC suspension and 25 µL of recipient plasma were added on top of the gel in successive card microtubes (6 microtubes per card) and incubated at 37 °C for 15 min in an incubator (Thermoline Scientific Refrigerated Incubator, Smithfield, New South Wales). Thereafter, the gel column cards were centrifuged for 10 min at 85 × g, in a centrifuge provided by the manufacturer (ID-Centrifuge 12 S II, DiaMed-ID, Microtyping System, DiaMed GmbH). An incompatibility score of 0 was given if all RBCs were seen at the bottom of the gel, ruling out agglutination. A score of 1+ was given if RBCs were seen in the bottom half of the gel, 2+ if RBCs were seen throughout the entire gel, 3+ if RBCs were seen in the top half of the gel, and 4+ if all RBCs were evident in a tight disc at the top of the gel. Consistent with literature in dogs[2] and with our previous study,[7] crossmatches were interpreted as compatible or negative, if less than or equal to 1+, and incompatible or positive if greater than or equal to 2+. Compatibility reactions were evaluated by a single experienced investigator (CRS). Each recipient was crossmatched against itself as a negative control, but positive controls were not performed as there was no way to determine a priori that any donor-recipient combination would be incompatible, as methods to blood type sheep are not currently available.

Results

Blood was collected from 21 dams; 172 individual dam-dam cross matches were performed. Two of these tests were incompatible (1.2%). In addition, 19 fetal blood samples collected immediately after cesarean delivery were crossmatched with 21 maternal samples to generate 174 maternal-fetal individual cross matches. No maternal-fetal incompatibility reactions were observed. Blood samples were collected from 4 fetuses at the end of the EVE experiment and crossmatched against 10 maternal samples. No incompatibilities were detected in this subset of tests. Negative control tests were all scored 0 (no incompatibility).

Discussion

The primary aim of this study was to investigate the incidence of incompatibility reactions between maternal RBCs and fetal plasma in sheep. The results demonstrated that all maternal donors and fetal recipients were compatible. In addition, in major cross matches performed using blood from adult ewes, the incidence of incompatibility was 1.2%. These data suggest that lambs are not born with antibodies against other blood types, but rather may acquire these antibodies at some time during early life. Furthermore, the risk of incompatibility reactions between ewes of a similar breed and from a single farm of origin is very low.

The current study found no incompatibility reactions between dams and fetuses. The majority of the tests were performed on fetal blood collected immediately after cesarean delivery. Results support the theory that antibodies to RBCs are not present at birth in lambs. A previous study reported that passively acquired antibodies (from colostrum) disappear before the lamb begins to produce its own antibodies, which may not be evident in the serum until 5 to 7 mo of age.[9] More specifically, lambs with an antigen designated R in their sera had RBCs that did not become R-positive until 16.4 d of age, while lambs with an antigen designated O in their serum had RBCs that become O-positive at 28.4 d of age.[9] Reference samples from lambs containing the antigens and antibodies (R antigen, anti-R antibodies, O-antigen, and anti-O antibodies) are not commercially available, making comparisons of current and historical findings difficult. Nevertheless, these data,[9] along with our current results, suggest that preterm lamb fetuses can accept donor blood with negligible risk of an immunologic transfusion reaction because they do not ingest colostrum and are too young to have produced their own antibodies against foreign RBC antigens. With respect to lambs that receive blood transfusions while on the artificial placenta, a small subset of crossmatching tests were performed. During the 5 d spent on this platform, the lambs received multiple transfusions of pRBCs and thawed fresh frozen plasma. Theoretically, these transfusions could introduce antibodies or sensitize the fetus to foreign antigens, but no incompatibilities were detected in this small subset (4 fetuses and 10 dams) of tests. Any given fetus received blood products from a single maternal source, which likely reduced the risk of incompatibility reactions.

The incidence of incompatibility reactions between dams in this study was low as compared with previous studies, which reported a frequency of incompatibility from 16% to 30.8%.[4,7,10] The size and structure of these studies varied, with 12 male Merino sheep in a prospective study,[10] 58 small ruminants in a retrospective clinical case series[4] and 8 Merino ewes in the most recent prospective study.[7] This variation in sample size, breed of sheep and methodology complicates meaningful comparison the results. However, the current prospective study with 21 dams provides some confidence that in our cohort of Merino sheep, the risk of transfusion reactions was very low. A limitation of the most recently published prospective study[7] is that the samples were not analyzed for 3 to 4 wk after collection; this period of storage possibly permitted sample degradation and false incompatibility reactions.[3,7] In the current study, samples were analyzed within 2 wk of collection; given the risk of increased incompatibility reactions after storage, the results of the current study may be less confounded by the relatively short duration of storage.

The results of this study can only be interpreted with consideration of a number of limitations to the scope and context of the study. First, a single breed of sheep was studied, and the breed of sheep may influence cross-compatibility. Second, further work investigating the antibody status of preterm lamb fetuses after a prolonged period on the artificial placenta may be warranted if the model is extended to longer periods of time (weeks). In our study, only a small number of tests were performed on lambs at the end of their short time on the artificial placenta. In addition, we could have performed both minor and major cross-matches. However, the focus of our study was to evaluate the risk of an acute immunologic hemolytic transfusion reaction as a result of transfusion of maternal red blood cells to the fetus. Major, rather than minor, crossmatching was therefore more relevant to the EVE model. Finally, lambs may be tolerant and therefore less likely to mount a potent immune response, as compared with adult sheep, which may give rise to false negative results. Nevertheless, in the context of the EVE model in which the animal of interest is the preterm lamb, the results are relevant.

We have previously recommended that the use of donor-recipient cross matching in sheep to facilitate identification of compatible blood products for transfusion.[7] Gel card crossmatching, as performed in the current study, is suitable to predict the compatibility of donor and recipient, as is the saline and albumin tube compatibility test that has been reported recently.[10] Our recommendation stands despite the promising results of the current study. In conclusion, in the context of the artificial placenta model in which lambs are supported for a limited period of time (days to weeks), the risk of transfusion reactions is very low if a single donor source is used for consecutive transfusions.

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