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Differences in amniotic amino acids concentrations between pregnancies obtained with transfer of vitrified/thawed IVP embryos and with natural mating in sheep

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Abstract

In vitro embryo production (IVP) and cryopreservation are associated with a high incidence of pregnancy complications and fetal abnormalities that may be linked with alterations of placental development. The amniotic fluid is partly derived from the transport of water and solutes across the placenta and provides the fetus with amino acids (AAs), which are the building blocks for biomolecules involved in physiologic growth and development. To better understand the anomalies associated with IVP pregnancies, the present study was conducted to test the hypothesis that amniotic concentrations of AAs differ in pregnancies derived from vitrified/thawed (V/T) IVP embryos compared to gestations obtained with natural mating (NM) in sheep. Amniotic fluid was sampled in ewes that were pregnant after transfer of V/T IVP embryos and that had conceived with natural mating between day 60 and 65 (V/T n=6; NM n=11) and between day 80 and 85 (V/T n=5; NM n=14) of gestation via ultrasound-guided amniocentesis. Concentrations of 16 AAs in the amniotic fluid were measured using high-

performance liquid chromatography. At day 60-65 of gestation concentrations of cystine, phenylalanine and isoleucine were lower in V/T compared to NM ewes. At day 80-85 of pregnancy the mean concentrations of cystine and lysine were lower in the V/T vs NM group. The total AA concentration per ewe was similar between groups at day 60-65 and 80-85 and decreased by 55% from day 60-65 to 80-85 of gestation in all ewes. The most abundant AA at day 60-65 of gestation was alanine in both groups, whereas at day 80-85 the most abundant AAs were alanine in NM and glycine in V/T ewes respectively; cystine was the less abundant detectable AA in all ewes at both stages of gestation. Results demonstrate that V/T IVP embryos have decreased concentrations of individual AAs in the amniotic fluid during the second trimester of gestation, possibly because of an impaired placental vasculogenesis or because of a reduced placental transport. These novel findings are relevant to unravel the mechanisms responsible for the issues of pregnancies achieved with the transfer of IVP and cryopreserved embryos.

Keywords: amniotic fluid; AA; *in vitro*; embryo; vitrification; ovine.

1. Introduction

Reproductive efficiency is fundamental for the economic sustainability of animal husbandry, including farming of small ruminants. To control and improve animal reproduction, assisted reproductive technologies (ART) have been developed, such as artificial insemination, multiple ovulation, embryo transfer and *in vitro* production of embryos (IVP) [1, 2]. The latter technique involves four major steps: oocyte collection (either from a live animal via ovum pick-

up or from ovaries recovered at slaughter), oocyte *in vitro* maturation, fertilization and development of the embryo up to the blastocyst stage. The embryos produced can be transferred fresh into the uterus of estrous-synchronized recipients or can be stored via cryopreservation and subsequently transferred after thawing. These different steps are now well established in domestic ruminant species (cattle, sheep, and goat) [3]. Nevertheless IVP is still an inefficient process in ruminants because the proportion of embryos reaching the transferable stage is rarely over 40% [4-6] and because the issue of abnormal pregnancies involving IVP embryos has not been satisfactorily resolved [7]. These problems hold true in IVP and embryo transfer in sheep, contributing heavily to limit the commercial application of these techniques. According to the international embryo transfer society in 2012 890,875 bovine embryos – produced *in vivo* or fertilized *in vitro* – were transferred, whereas 12,458 *in vivo* derived ovine embryos were transferred [8]. Modern ART, such as IVP, embryo cryopreservation and embryo transfer, are not routinely applied in sheep breeding programs, but are only used for experimental purposes [9]. Hence, more research is needed to understand why, following transfer, IVP embryos perform considerably poorer than *in vivo* derived embryos and have frequently been associated with fetal and neonatal abnormalities [7].

The developing fetus is surrounded by the amniotic sac containing the amniotic fluid which derives, in part, from (1) the transport of water and solutes across the placenta from the endometrium and (2) uterine secretions [10]. The volume and composition of the amniotic fluid are strictly regulated during gestation, reflecting the dynamic balance maintained through exchange between maternal circulation and fetal environment [11, 12]. The fetus may actively

drink the amniotic fluid; it represents a reservoir of water and proteins that can influence fetal-placental development and functions [10]. The amniotic fluid also contains amino acids (AAs), which are the building blocks for biomolecules involved in physiologic growth and development, and precursors of nitrogenous substances with varied regulatory functions [13]. There is now compelling evidence indicating that the disruption of placental development during the initial pregnancy phases is involved in the developmental failure of embryos from different species, as well as the abnormal phenotypes of pregnancies obtained by ART [7, 14, 15]. The present study was conducted to test the hypothesis that concentrations of AAs in the amniotic fluids differ in pregnancies derived from vitrified/thawed (V/T) IVP embryos compared to gestations obtained with natural mating (NM) in sheep.

2. Materials and methods

All animal experiments were performed in accordance with DPR 27/1/1992 (Animal Protection regulations of Italy) in conformity with European Community regulation 86/609.

2.1 Vitrified/Thawed (V/T) group

2.1.1 In vitro embryo production

Ovaries of Sarda ewes were collected from a local slaughterhouse, oocytes were recovered, and embryos were produced *in vitro* as previously described [16]. When not specified, products were purchased from All chemicals, Sigma Chemical Co. (St. Louis, MO). Briefly, adult (4-6 years old) ovine ovaries were transported to the laboratory within 1-2 h in Dulbecco Phosphate Buffered Saline (PBS) with penicillin (100 µg/mL) and streptomycin (100 µg/mL).

Collection of cumulus-oocyte complexes (COCs) was performed in sterile Petri dishes containing 20 mM Hepes-buffered TCM 199 supplemented with 0.1% (w/v) polyvinyl alcohol and antibiotics (0.1 g/L penicillin and 0.1 g/L streptomycin). Only COCs showing several intact cumulus cell layers and compacted cytoplasm were selected and matured *in vitro* in TCM 199 supplemented with 10% heat-treated estrus sheep serum, 0.1 IU/mL FSH and 0.1 IU/mL LH (Pergonal, Serono, Italy) and 100 μM cysteamine. Thirty to thirty-five COCs were cultured for 24 h in 5% CO_2 in air at 38.5°C in four-well petri dishes (Nunclon, Nalge Nunc International, Denmark) with 600 μL of maturation medium, layered with 300 μL of mineral oil. *In vitro* matured oocytes were fertilized in SOF medium with 2% heat-treated estrus sheep serum for 22 h at 38.5°C and 5% CO_2, 5% O_2 and 90% N_2 atmosphere in four-well petri dishes (Nunclon). Frozen-thawed spermatozoa obtained from pooled ejaculations of a single ram (Sarda breed) selected by swim-up technique ($1 \cdot 10^6$ spermatozoa/mL) under mineral oil were used for IVF. Presumptive zygotes were transferred separately and cultured for 7 days in four well Petri dishes containing SOF + essential and non-essential amino acids at oviductal concentration [17] + 0.4% bovine serum albumin (BSA) under mineral oil, in maximum humidified atmosphere with 5% CO_2, 5% O_2, 90% N_2 at 39°C. The first cleavage was registered between 24-26 h after the start of fertilization.

2.1.2 Embryo vitrification and thawing

Expanded blastocysts were recovered on the 7^{th} day of culture and vitrified in Cryotop devices (Kitazato Ltd., Tokyo, Japan) using the minimum essential volume method [18]. Briefly, groups of 2/3 blastocysts were initially equilibrated for 1 min at room temperature in holding medium (HM) consisting of 20 mM Hepes-buffered TCM-199 supplemented with 20% fetal

116 calf serum. Embryos were then incubated in HM supplemented with 7.5% ethylene glycol
117 (EG) and 7.5% dimethyl sulfoxide (DMSO) for 3 min and successively transferred into HM
118 with 16.5% EG, 16.5% DMSO and 0.25 M sucrose for 20 sec. Blastocysts were loaded on
119 Cryotop devices with a small volume of the latter solution (<2.0 µL) and immediately plunged
120 into liquid nitrogen for storage. Embryos were thawed by placing Cryotops in HM with 1.25 M
121 sucrose for 1 min at room temperature. Recovered blastocysts were sequentially transferred
122 into HM with 0.5 M sucrose for 3 min, then into HM with 0.25 M sucrose for 3 min and in HM
123 for 1 h.
124
125 *2.1.3 Embryo transfer*
126 Eleven adult (3–5 years old) Sarda sheep were housed at the experimental facilities of the
127 Department of Veterinary Medicine at the University of Sassari (Italy) and fed a live-weight
128 maintenance ration throughout the experimental procedure. Estrous cycles were
129 synchronized using intravaginal progestagen impregnated sponges (45 mg fluorogestone
130 acetate sponges, Cronogest®, Intervet S.r.l., Italy) for 14 days and a single administration of
131 333 IU/ewe of eCG (Folligon®, Intervet S.r.l., Italy) on the day of the sponge removal. Twenty-
132 four hours after sponge removal a teaser ram was introduced in the flock to detect estrus.
133 Seven days after the onset of estrus, three vitrified/thawed blastocysts were surgically
134 transferred into each recipient via median laparatomy. All procedures were carried out under
135 epidural anesthesia (3.5 mg/kg, Lidocaina 2%®, Pfizer, Italy). The recipients were secured in
136 a cradle in dorsal recumbency. The uterus was exposed throughout the laparotomic incision
137 and both ovaries were exteriorized in order to assure the presence of at least one active
138 corpus luteum. Embryos were transferred with as little as 0.1 mL medium (PBS–BSA) using a

plastic catheter inserted at the major curvature of the uterine horn on the same side of the ovary bearing a corpus luteum. After surgery, a single dose of amoxicillin (12.5 mg/kg, Longocillina L.A. ®, Ceva, Italy) was administered to the ewes. Pregnancy diagnosis was performed 15 days after surgery by transabdominal ultrasonography (A6V®, Sonoscape Ltd.), using a 9-4 MHz micro-convex probe and eleven ewes were identified as pregnant with a single fetus. No twin pregnancies were identified. Amniocentesis was subsequently performed on six ewes at Day 60-65 and on five ewes at Day 80-85 of gestation.

2.2 Naturally mated group (NM)

Adult (3–5 years old) Sarda ewes were housed in a commercial farm in the province of Sassari (Italy) and fed a live-weight maintenance ration throughout the experiment. Estrous cycles were synchronized as described above. Fertile rams of the same Sarda breed bearing a marking harness were introduced in the flock for the breeding season and marked ewes were identified as mated. Transabdominal ultrasonography was performed 21 days after mating to identify ewes pregnant with a single fetus and was repeated on pregnant females starting approximately 50 days after mating to estimate fetal age [19, 20]. Eleven ewes that were 60-65 days pregnant and 14 ewes that were 80-85 days pregnant with a single fetus were identified.

2.3 Amniocentesis and amino acid analysis in the amniotic fluid

To sample the amniotic fluid, on day 60-65 (V/T n = 6, NM n = 11) and on day 80-85 (V/T n = 5, NM n = 14) of gestation trans-abdominal ultrasound-guided amniocentesis was performed (A6V®, Sonoscape Ltd.) using a 9-4 MHz micro-convex probe. Ewes were secured in a cradle in dorsal recumbency, shaved and the area between the head of the fetus and the membrane of the amniotic sac was identified via ultrasonography as the site of puncture. The area was accurately disinfected and the ultrasound transducer was covered with a sterile glove. A 22 ga sterile needle was placed on one side of the transducer and was inserted under direct ultrasound visualization until the tip of the needle was at the center of the area between the head of the fetus and the amniotic membrane. The needle was connected to an extension set and a syringe and 10 mL of amniotic fluid were collected.

At term, ewes in both groups lambed single live healthy lambs whose weight was within range for the Sarda breed (3-4 kg).

2.4 High-pressure liquid chromatography

Amniotic fluid samples were centrifuged at 4000 *g* for 10 min and the supernatant was stored at -80°C for subsequent analysis by high-pressure liquid chromatography [21].

Concentrations of the following amino acids were measured in the amniotic fluid: alanine, arginine, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine. Amino acids concentrations were determined using an Acquity ultra-performance liquid chromatography (UPLC) system equipped with an Acquity UPLC Photodiode Array (PDA) eλ Detector (Waters Corporation, Milford, MA, USA). Amino acids derivatization was performed using an AccQ-Tag Ultra

Derivatization Kit following the manufacturer's instructions (Waters Corporation, Milford, MA, USA). Once derivatized, labeled AAs were separated on a 2.1 x 100 mm, 1.7 µm AccQ-Tag Ultra column and UV detected by the PDA detector set at 260 nm.

2.5 Statistical analysis

All results are expressed as mean ± standard error of the means. Statistical analysis was conducted with R-software version 3.03. The Shapiro-Wilk test of normality was performed. Data were not normally distributed, hence data were $\log_{10}$ transformed before analysis. Analysis of variance was used to test whether total and individual concentrations of amino acids differed between groups and day of gestation. Statistical significance was set at a probability value of 0.05 or less.

3. Results

At day 60-65 of gestation the total amino acid concentration per ewe was similar between groups (Fig 1), but the mean concentrations of cystine, phenylalanine and isoleucine were lower in V/T compared to NM ewes (Fig 2a).The mean total amino acid concentration decreased by 55% from day 60-65 to 80-85 of gestation in all ewes, but the decrease was greater in IVP embryo transferred compared to naturally mated ewes (65% vs 52% respectively; Fig 1). At day 80-85 of pregnancy no difference was detected between groups in the total amino acid concentration (Fig 1), whereas the mean concentrations of cystine and lysine (Fig 2b) were lower in the V/T vs the NM ewes (Fig 2b). At day 80-85 phenylalanine, isoleucine, methionine, tyrosine, histidine were undetectable in four V/T ewes, hence these

amino acids were excluded from the analysis. The most abundant amino acid at day 60-65 of gestation was alanine in both groups, whereas at day 80-85 the most abundant amino acids were alanine in NM and glycine in V/T ewes respectively; cystine was the less abundant detectable amino acid in all ewes at both time points (Fig 2).

4. Discussion

This study reports lower concentrations of individual AAs in the amniotic fluid of pregnancies achieved after the transfer of cryopreserved IVP embryos compared to gestations obtained with natural mating. Individual concentrations of the majority of AAs detected were lower in V/T vs NM. However, a statistically significant difference was not detected in all comparisons; this could be due to the limited number of samples.

The amniotic fluid is composed of water and electrolytes from both the fetus (kidneys, lungs, epidermis and fetal blood vessels in the placenta and umbilical cord) and the mother (decidual blood vessels via amniotic membranes) [10]. The AAs in the amniotic fluid mainly derive from maternal and fetal circulation through the placenta [22, 23]; they are transported across the placenta by active transport mediated by numerous different amino acid transporters [24]. In the present study the lower concentration of individual AAs in the amniotic fluid of V/T pregnancies could be caused by an impaired placental vascularization. We speculate this because in a recent study a severe impairment vasculogenesis early in gestation was observed in pregnancies obtained through IVP from oocytes of FSH-treated ewes, as assessed by a reduction in the number and diameter of placental vessels and an

underdevelopment of the cardiovascular systems in IVP vs naturally conceived conceptuses [25]. Reduced placental vascularization and expression of angiogenic factors in fetal membranes were also detected early in pregnancies after transfer of embryos obtained with *in vitro* fertilization [15]. In our study, the transfer of V/T embryos may have impaired placental perfusion and consequently the passage of AAs from maternal circulation to fetal fluids.

Another potential cause of the lower amniotic individual AAs concentrations in V/T pregnancies may be the reduction of the AAs transport systems in the placenta. Compelling evidence in humans and sheep shows that in intra-uterine growth restricted pregnancies placental transport of essential AAs, such as leucine, lysine and threonine, is reduced because a number of placental transport systems are down-regulated [26, 27]. Similarly, a reduction in the AAs transport systems may have caused the low amniotic individual AAs concentrations in V/T gestations reported in the present study. Amino acid transport systems are either highly specific or have a broad specificity for a group of AAs [28]. For example, one transporter can transfer a certain kind of AA (e.g., system A can transfer neutral amino acids, like alanine, serine, proline and glutamine) [29]. In contrast, other transport systems have overlapping substrate specificity. Nevertheless, the fact that in this study the concentrations of different AAs were reduced suggests a broad effect on different transport systems rather than the down-regulation of a single one.

The AAs that were reduced in V/T pregnancies compared to controls were isoleucine, phenylalanine, lysine and cystine. Isoleucine is a branded-chain AA, such as leucine and valine; they have a positive effect on protein synthesis in skeletal muscle [30] and donate the

amino group for synthesis of glutamine in mammals, which plays a prominent role in fetal carbon and nitrogen metabolism [31]. Phenylalanine is an aromatic AA together with tyrosine, histidine, and tryptophan. They are precursors of neurotransmitters, regulating neurological functions during embryonic and fetal development and alterations of these AAs are proposed to exacerbate neuronal viability [32]. Interestingly all aromatic AAs analyzed were undetectable at day 80-85 of gestation in the majority of V/T ewes. Cystine is one of the precursors of glutathione and its supply is a rate-limiting factor in its synthesis in humans [33]; glutathione is vital for antioxidant reactions and is important in the regulation of immune functions [34]. Lysine is widely supplemented in animal feed, especially for monogastric animals like broilers, poultry and swine and as a supplement for humans, to improve the feed quality by increasing the absorption of other amino acids [35]. Taken together these findings suggest that pregnancies obtained from V/T embryos may have low availability of important AAs and this could in turn impair conceptus development, having long term consequences on the newborn.

All gestations in this study ended with the birth of a healthy lamb whose weight was within the normal range for the Sarda breed. This may mean either that the decrease in individual amniotic AAs levels was not severe enough to cause pregnancy complications or that concentrations of AAs may have increased later in gestation. Also, we cannot exclude that non-viable pregnancies from V/T may have even lower amniotic AAs concentrations. For example, in a recent study amniotic AA levels were different in non-viable pregnancies achieved after somatic cell nuclear transfer compared to IVP gestations, but IVF and viable SCNT pregnancies had similar amniotic AA concentrations [12]. Thus, measuring amniotic

AAs concentrations in pregnancies with placental and fetal developmental defects that often result from the transfer of IVP embryos [7] may contribute to better understand placental transport of AAs in IVP pregnancies. Similarly the comparison of amniotic AAs concentrations in pregnancies resulting from the transfer of fresh IVP embryos vs cryopreserved IVP blastocyst merits further research to distinguish potential detrimental effects of blastocyst cryopreservation. The stage of pregnancy at which amniocentesis is performed is also important. We selected day 60-65 and 80-85 of gestation because amniocentesis can be performed safely; placental development is completed approximately by day 60 of gestation in sheep [36], but after the third month fetal movements increase the risk of fetal/membranes damages.

Different sires were used to produce embryos *in vitro* and for natural mating. Since the sire contributes to the development of the conceptus and of fetal membranes, we cannot exclude that this may have influenced our results. Nevertheless, a sire effect would have been relevant if a single female had been used for oocyte retrieval and natural mating, whereas in our study, oocytes were collected from ovaries of several different females and other ewes were used to obtain pregnancies with natural mating. The validity of this model is confirmed by the fact that it was used in previously mentioned studies; placental vasculogenesis and epigenetic regulation of gene expression during placentogenesis were compared in pregnancies obtained from transfer of *in vitro* produced embryos vs natural mating using Sarda breed sheep and different rams for IVP and natural mating [14, 25]. Also, only Sarda sheep were used in our study and rams were all registered in the official breed herd book. Weight at birth in this breed is quite constant [37], hence it is highly unlikely that the use of

different rams influenced fetal and placental development, and consequently amniotic AA concentration.

Alanine, glycine, glutamic acid and serine were the most abundant AAs in both experimental groups, in accordance with what was reported in naturally mated ewes [11] and in IVP bovine pregnancies [12]. Also, the dramatic twofold decrease in amniotic AA concentration observed from day 60 to 80 of gestation is similar to what was previously reported in naturally mated ewes [11], but our data show a more pronounced, albeit not significant, decrease in V/T pregnancies compared to controls. This intriguing finding indicates that the impairment in placental transport of AAs and consequently their availability may further worsen as gestation progresses.

In conclusion, results demonstrate that V/T IVP embryos have decreased concentrations of individual AAs in the amniotic fluid during the second trimester of gestation, possibly because of an impaired placental vasculogenesis or of a reduced placental transport. These findings provide the basis for future studies to elucidate mechanisms responsible for the large range of developmental abnormalities associated with the transfer of IVP and cryopreserved embryos.

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References

[1] Amiridis GS, Cseh S. Assisted reproductive technologies in the reproductive management of small ruminants. Anim Reprod Sci. 2012;130:152-61.

[2] Cognié Y, Poulin N, Locatelli Y, Mermillod P. State-of-the-art production, conservation and transfer of in-vitro-produced embryos in small ruminants. Reprod Fertil Dev. 2004;16:437-45.
[3] de Souza-Fabjan JM, Panneau B, Duffard N, Locatelli Y, de Figueiredo JR, Freitas VJ, et al. In vitro production of small ruminant embryos: late improvements and further research. Theriogenology. 2014;81:1149-62.
[4] Lonergan P, Fair T. In vitro-produced bovine embryos: dealing with the warts. Theriogenology. 2008;69:17-22.
[5] Rizos D, Fair T, Papadopoulos S, Boland MP, Lonergan P. Developmental, qualitative, and ultrastructural differences between ovine and bovine embryos produced in vivo or in vitro. Mol Reprod Dev. 2002;62:320-7.
[6] Cognié Y, Baril G, Poulin N, Mermillod P. Current status of embryo technologies in sheep and goat. Theriogenology. 2003;59:171-88.
[7] Farin PW, Piedrahita JA, Farin CE. Errors in development of fetuses and placentas from in vitro-produced bovine embryos. Theriogenology. 2006;65:178-91.
[8] Perry G. 2012 Statistics of embryo collection and transfer in domestic farm animals. Data retrieval committee reports: International Embryo Transfer Society; 2013.
[9] Carta A, Casu S, Salaris S. Invited review: Current state of genetic improvement in dairy sheep. J Dairy Sci. 2009;92:5814-33.
[10] Bazer F. Allantoic fluid: regulation of volume and composition. In: Brace RA, MG R, JE R, editors. In Reproductive and perinatal medicine: Fetal and neonatal body fluids. Ithaca, USA: Perinatology Press; 1989. p. 135-55.
[11] Kwon H, Spencer TE, Bazer FW, Wu G. Developmental changes of amino acids in ovine fetal fluids. Biol Reprod. 2003;68:1813-20.
[12] Zhou W, Gosch G, Guerra T, Broek D, Wu G, Walker S, et al. Amino acid profiles in first trimester amniotic fluids of healthy bovine cloned pregnancies are similar to those of IVF pregnancies, but not nonviable cloned pregnancies. Theriogenology. 2014;81:225-9.
[13] Wu G. Amino acids: metabolism, functions, and nutrition. Amino Acids. 2009;37:1-17.
[14] Ptak GE, D'Agostino A, Toschi P, Fidanza A, Zacchini F, Czernik M, et al. Post-implantation mortality of in vitro produced embryos is associated with DNA methyltransferase 1 dysfunction in sheep placenta. Hum Reprod. 2013;28:298-305.
[15] Grazul-Bilska AT, Johnson ML, Borowicz PP, Bilski JJ, Cymbaluk T, Norberg S, et al. Placental development during early pregnancy in sheep: effects of embryo origin on vascularization. Reproduction. 2014;147:639-48.
[16] Bebbere D, Bogliolo L, Ariu F, Fois S, Leoni GG, Succu S, et al. Different temporal gene expression patterns for ovine pre-implantation embryos produced by parthenogenesis or in vitro fertilization. Theriogenology. 2010;74:712-23.
[17] Walker SK, Hill JL, Kleemann DO, Nancarrow CD. Development of ovine embryos in synthetic oviductal fluid containing amino acids at oviductal fluid concentrations. Biol Reprod. 1996;55:703-8.
[18] Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. Theriogenology. 2007;67:73-80.
[19] Aiumlamai S, Fredriksson G, Nilsfors L. Real-time ultrasonography for determining the gestational age of ewes. Vet Rec. 1992;131:560-2.
[20] Nieddu S, Strina A, Corda A, Furcas G, Ledda M, Pau Salvatore, et al. Comparation of Foetal Growth in Natural Mated and Vitrified/Warmed Ovine Embryos by Ultrasonographic Measurements. Veterinaria. 2014:91-7.
[21] Zhang S, Geng H, Xie H, Wu Q, Ma X, Zhou J, et al. The heterogeneity of cell subtypes from a primary culture of human amniotic fluid. Cell Mol Biol Lett. 2010;15:424-39.
[22] Baetz AL, Hubbert WT, Graham CK. Developmental changes of free amino acids in bovine fetal fluids with gestational age and the interrelationships between the amino acid concentrations in the fluid compartments. J Reprod Fertil. 1975;44:437-77.

[23] Regnault TR, de Vrijer B, Battaglia FC. Transport and metabolism of amino acids in placenta. Endocrine. 2002;19:23-41.
[24] Cariappa R, Heath-Monnig E, Smith CH. Isoforms of amino acid transporters in placental syncytiotrophoblast: plasma membrane localization and potential role in maternal/fetal transport. Placenta. 2003;24:713-26.
[25] Fidanza A, Toschi P, Zacchini F, Czernik M, Palmieri C, Scapolo P, et al. Impaired placental vasculogenesis compromises the growth of sheep embryos developed in vitro. Biol Reprod. 2014;91:21.
[26] Jansson T, Scholtbach V, Powell TL. Placental transport of leucine and lysine is reduced in intrauterine growth restriction. Pediatr Res. 1998;44:532-7.
[27] Paolini CL, Marconi AM, Ronzoni S, Di Noio M, Fennessey PV, Pardi G, et al. Placental transport of leucine, phenylalanine, glycine, and proline in intrauterine growth-restricted pregnancies. J Clin Endocrinol Metab. 2001;86:5427-32.
[28] Lin G, Wang X, Wu G, Feng C, Zhou H, Li D, et al. Improving amino acid nutrition to prevent intrauterine growth restriction in mammals. Amino Acids. 2014.
[29] Novak DA, Beveridge MJ, Malandro M, Seo J. Ontogeny of amino acid transport system A in rat placenta. Placenta. 1996;17:643-51.
[30] Davis TA, Fiorotto ML, Burrin DG, Reeds PJ, Nguyen HV, Beckett PR, et al. Stimulation of protein synthesis by both insulin and amino acids is unique to skeletal muscle in neonatal pigs. Am J Physiol Endocrinol Metab. 2002;282:E880-90.
[31] Lin G, Liu C, Feng C, Fan Z, Dai Z, Lai C, et al. Metabolomic analysis reveals differences in umbilical vein plasma metabolites between normal and growth-restricted fetal pigs during late gestation. J Nutr. 2012;142:990-8.
[32] van Vliet E, Eixarch E, Illa M, Arbat-Plana A, González-Tendero A, Hogberg HT, et al. Metabolomics reveals metabolic alterations by intrauterine growth restriction in the fetal rabbit brain. PLoS One. 2013;8:e64545.
[33] Grimble RF. The effects of sulfur amino acid intake on immune function in humans. J Nutr. 2006;136:1660S-5S.
[34] Margonis K, Fatouros IG, Jamurtas AZ, Nikolaidis MG, Douroudos I, Chatzinikolaou A, et al. Oxidative stress biomarkers responses to physical overtraining: implications for diagnosis. Free Radic Biol Med. 2007;43:901-10.
[35] Anastassiadis S. L-lysine fermentation. Recent Pat Biotechnol. 2007;1:11-24.
[36] Wu G, Bazer FW, Datta S, Johnson GA, Li P, Satterfield MC, et al. Proline metabolism in the conceptus: implications for fetal growth and development. Amino Acids. 2008;35:691-702.
[37] Vacca GM, Carcangiu V, Dettori ML, Pazzola M, Mura MC, Luridiana S, et al. Productive performance and meat quality of Mouflon×Sarda and Sarda×Sarda suckling lambs. Meat Sci. 2008;80:326-34.

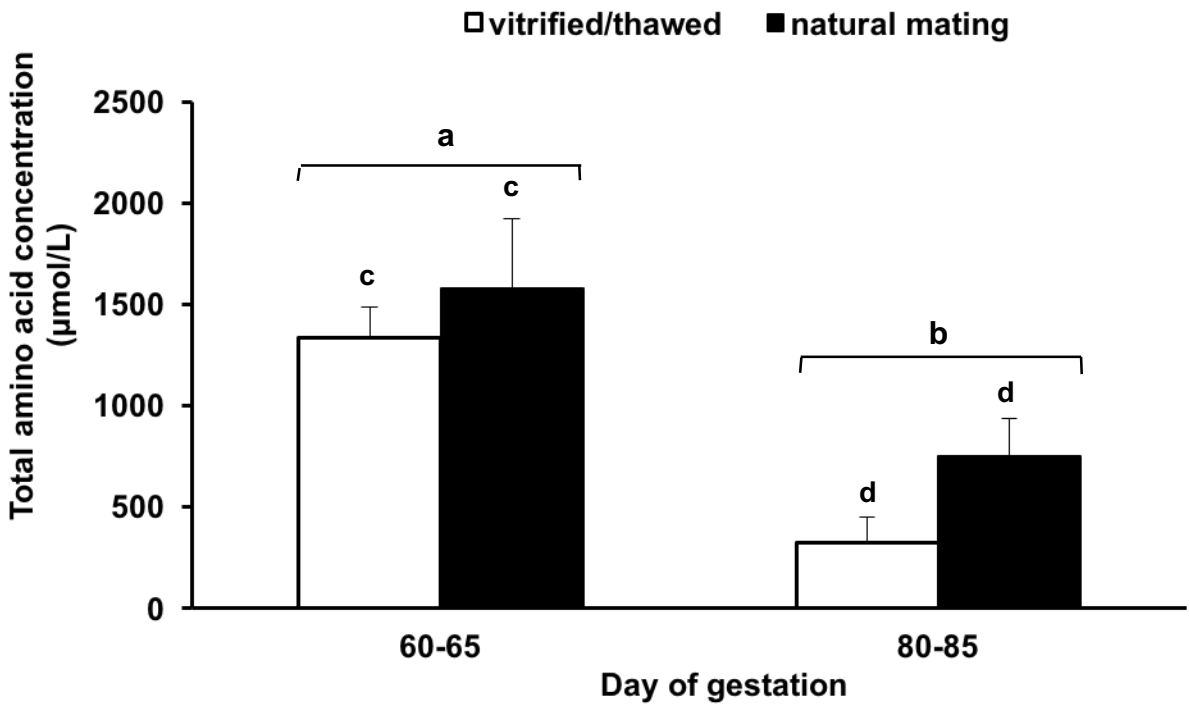

Fig. 1. Total amino acid concentration per ewe in the amniotic fluid of ewes that were naturally mated (NM) or embryo transferred with IVP vitrified/thawed embryos (V/T) at Day 60-65 (V/T = 6; NM = 11) and Day 80-85 (V/T = 5; NM = 14) of gestation. Data are expressed as means ± SEM. Different letters indicate statistical difference (a vs b $P<0.001$; c vs d $P<0.05$).

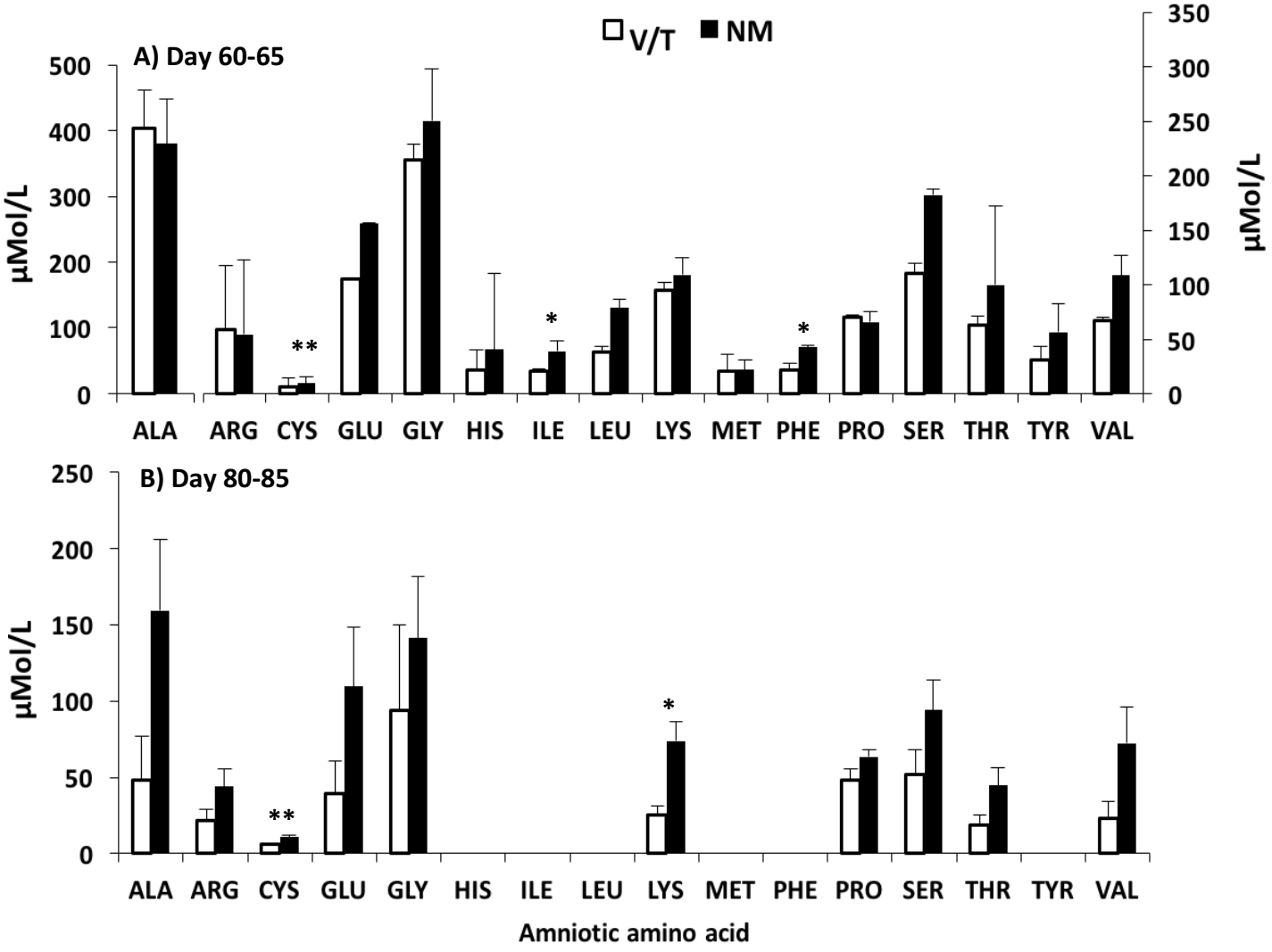

Fig. 2. Concentrations of single amino acids in the amniotic fluid in ewes that were naturally mated (NM) or embryo transferred with *in vitro* produced vitrified/thawed embryos (V/T) at day 60-65 of gestation (Panel A; V/T = 6, NM n = 11) and at day 80-85 of gestation (Panel B; V/T = 5, NM n = 14). Data are expressed as means ± SEM. Different superscripts indicate statistical differences between groups within the same panel (* P<0.05; ** P<0.03). Abbreviations: ALA=alanine; ARG=arginine; CYS=cystine; GLU=glutamic acid; GLY=glycine; HIS=histidine; ILE=isoleucine; LEU=leucine; LYS=lysine; MET= methionine;

444 PHE=phenylalanine; PRO= proline; SER= serine; THR=threonine; TYR= tyrosine; VAL=

445 valine.