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Determination of Biochemical Changes in Amniotic Fluid According to the Number of Offspring in Awassi Sheep

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ABSTRACT

Objective: The present study was carried out to investigate the difference in metabolic needs between twin and singleton pregnancies by looking at some biochemical and hormonal markers in the amniotic fluid taken at the time of birth in Awassi sheep. **Materials and Methods:** A total of 30 Awassi sheep were used in the study. The first group of the study (Group 1, n=15); ewes carrying one offspring included the second group of the study (Group 2, n=15); created sheep carrying two offsprings. From the sheep in both study groups; 10 ml of amniotic fluid was taken with a sterile syringe without disturbing the integrity of the amniotic sac hanging from the lips of the vulva at the time of delivery. Electrolyte (sodium, potassium, chlorine, calcium, phosphorus), total protein, glucose, liver (ALT and AST), kidney biomarkers (urea and keratinin) and cortisol levels were measured from the amniotic fluid. Obtained data were analyzed with independent samples t-test. **Results**: Glucose and cortisol levels were significantly higher in the twin pregnant group than in the singleton pregnant group ($p<0.05$). Calcium level was found to be significantly higher in the single pregnant group than in the twin pregnant group ($p<0.05$). As a result, a difference was observed in the parameters evaluated depending on the number of offspring in the amniotic fluid. **Conclusion:** It was concluded that considering the metabolic needs differences due to the number of offspring may be a parameter that should be evaluated in the follow-up of the pregnancy and birth process**.**

Keywords: Amniotic Fluid, Twin Pregnancy, Biochemical Markers, Cortisol

İvesi Irkı Koyunlarda Yavru Sayısına Göre Amniyon Sıvısındaki Biyokimyasal Değişikliklerin Belirlenmesi

ÖZ

Amaç: Sunulan çalışma İvesi ırkı koyunlarda doğum zamanı alınan amniyon sıvısı içerisindeki bazı biyokimyasal ve hormonal belirteçlere bakarak ikiz ve tekiz gebelikler arasındaki metabolik ihtiyaç farkını araştırmak için yapıldı. **Gereç ve Yöntem:** Çalışmada toplam 30 adet İvesi ırkı koyun kullanıldı. Çalışmanın birinci grubunu (Grup 1, n=15); tek yavru taşıyan koyunlar, çalışmanın ikinci grubunu (Grup 2, n=15) ise; iki yavru taşıyan koyunlar oluşturdu. Her iki çalışma gruplarındaki koyunlardan; doğum zamanı vulva dudakları arasından sarkan amniyon kesesinin bütünlüğünü bozmadan steril enjektör ile 10 ml amniyon sıvısı alındı. Alınan amniyon sıvısından elektrolit (sodyum, potasyum, klor, kalsiyum, fosfor), total protein, glikoz, karaciğer (ALT ve AST), böbrek biyomarkerları (üre ve keratinin) ve kortizol düzeyleri ölçüldü. Elde edilen veriler bağımsız gruplar t test ile analiz edildi. **Bulgular:** Glikoz ve kortizol düzeyleri ikiz gebe grubunda tekiz gebe grubuna göre anlamlı derecede yüksekti ($p<0.05$). Kalsiyum düzeyi tekiz gebe grubunda ikiz gebe grubuna göre anlamlı derecede yüksek olduğu görüldü ($p<0.05$). **Sonuç:** Yavru sayısına bağlı meydana gelen metabolik ihtiyaç farklılıklarının göz önüne alınması, gebelik ve doğum sürecinin takibinde değerlendirilmesi gereken bir parametre olabileceği kanısına varıldı.

Anahtar kelimeler: Amniyon Sıvısı, İkiz Gebeliği, Biyokimyasal Belirteçler, Kortizol

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INTRODUCTION

With the onset of implantation, structures originating from the trophecdoderm and turning into functional membranes over time form the fetal sacs. These membranes are from the outside to the inside; chorion, allontois and amnion (Alaçam, 1994; Assis Neto et al., 2010; Assis Neto, Santos, Pereira & Miglino 2009; Küplülü, 1990; Pfarrer et al., 2001; Rici et al., 2011; Schlafer, Fisher & Davies, 2000). Amniotic membrane; Starting from the umbilical cord, it is the innermost sac that is spherical and taut at the beginning of the pregnancy and extends according to the shape of the uterus and the offspring as the pregnancy progresses. Amniotic fluid; It is mucoid, in varying amounts depending on the type of domestic animals, and is formed by the filling of this fluid into the sac by diffusion of fluids from the mother's blood as well as fetal nasopharyngeal secretions. There is also a very small amount of fetal urine in the amniotic fluid. This fluid prevents the fetus from sticking to the mother, protects the offspring from physical impacts, and provides an energy source for the fetus in the early stages of pregnancy. In birth, it plays an important role in the lubrication of the birth canal (Alaçam, 1994; Küplülü, 1990).

Many adaptation mechanisms develop during pregnancy and these mechanisms change according to the course of pregnancy (Lain & Catalano, 2007). Pregnancy-related changes are affected by the number of developing fetuses (Mackie et al. 2019). 13-16 weeks of pregnancy in cattle and sheep. The amniotic sac is formed on the first day and then the amniotic fluid fills the amniotic sac (Robert 1986). The metabolic profile, metabolite synthesis/degradation of amniotic fluid originating from maternal, fetal and placental tissues are clear indicators of fetal maturation (especially kidneys and lungs) and biochemical changes. The metabolic composition of blood and amniotic fluid reflects these biochemical changes. (Briese et al., 1999; Underwood, Gilbert & Sherman. 2005). The biochemical profile of fetal fluids is affected by fetal metabolic and secretory activities as well as feto-maternal exchange via the placenta (Banan Khojasteh, Khadjeh, Ranjbar & Salehi, 2001; Essawi, Mostafa & El Shorbagy, 2020). Amniotic fluid is extremely important in understanding fetal metabolism and determining pathological conditions during pregnancy (Prestes, Chalhoub, Lopes & Takahira, 2001). Examination of the biochemical profile of the amniotic fluid is useful for the follow-up of the developing fetus (Moghaddam & Olfati, 2012). The biological systems of twin pregnant goats need homeostatic adaptation to meet the expected additional demands of twin pregnancies for the survival of mother and fetus. Metabolic changes during pregnancy, which may be associated with organic structural changes, are evaluated by measuring some biochemical markers and comparing them with reference values (Cuckle 2014). There is little information about the reproductive characteristics of sheep, especially with regard to the composition of the amniotic fluid. In our study, it was aimed to determine the metabolic need difference between twin and singleton pregnancies by measuring some biochemical and hormonal markers in the amniotic fluid of Awassi sheep and to have a problem-free pregnancy period by arranging the care and feeding conditions accordingly.

MATERIALS AND METHODS

Animal selection and experimental protocol

The presented study was carried out in the month of August-February, at the Harran University Faculty of Veterinary Medicine Practice Farm in Eyyubiye district of Şanlıurfa. The animal material of the study consisted of 30 Awassi sheep, 2-4 years old, who gave birth at least once, and who did not have any genital tract problems. Sheep were housed together until birth, and their eding and water needs were met adlibitum. Progesterone-based estrus synchronization was applied during the breeding season to aggregate births within certain periods. The progesterone-containing vaginal sponge was inserted into the vagina to remain in the vagina for 12 days (day 0). On the 11th day of administration, 2 ml of PGF2a (10 mg dinoprost tromethamine, Dinolytic, Pfizer) was administered intramuscularly. On the 12th day of the application, the vaginal sponge was removed and 500 IU PMSG (Ovagen PMSG, Bionhe) was injected intramuscularly. After PMSG injection, oestrus monitoring was carried out with a search ram for 30 minutes at 8-hour intervals for 3 days, and the estrus sheep were hand-crossed with rams with predetermined fertility. Pregnancy and twin/singleness examination were performed transrectally with linear array probe at 5 MHz frequency with real time ultrasound on the 35 th day after breeding sheep. Sheep were divided into 2 groups according to the number of offspring by ultrasonography. The first group of the study (Group 1, n=15); ewes carrying one offspring included the second group of the study (Group 2, n=15); created sheep carrying two offsprings. One week before the expected due date, the sheep were taken into separate sections and their births were followed up. At the end of the first stage of labor, 10 ml of amniotic fluid was taken with a sterile syringe without disturbing the integrity of the amniotic sac hanging between the lips of the vulva. The collected amniotic fluid was centrifuged at 3000 rpm for 10 minutes and analyzed.

Evaluation of biochemical and hormonal changes in amniotic fluid

Sodium (Na, mmol/L), potassium (K, mmol/L), chloride (Cl, mmol/L), phosphorus (P, mmol/L) and calcium (Ca, mg/dL) electrolytes, total protein, glucose, kidney (urea and creatinine) and liver marker (Alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) measurements were made using a biochemistry analyzer (SMT-120V, Chengdu Seamaty Technology™, China). Amniotic fluid cortisol level was determined spectrophotometrically (Molecular Device SpectraMax M5 Plate Reader, Pleasanton, CA, USA) using a commercial kit (Sheep Cortisol ELISA Kit, Mybiosource, USA).

Statistical analysis

Statistical analysis of the data was performed using the Statistical Package for the Social Sciences (SPSS for Windows; version 24.0) packaged software. The conformity of the variables to normal distribution was

examined using visual (histogram and Q-Q Plot) and analytical methods (Shapiro-Wilk tests). Descriptive analyses were reported as mean ± standard error of the mean (SEM) values for normally distributed variables. Since the data showed conformity to normal distribution, they were compared between groups using the Independent Samples t-test. The homogeneity of variances was determined using the Levene test. A value of $p<0.05$ was accepted as statistically significant for all analyses

Ethical considerations

This study was carried out based on the permission of Harran University Animal Experiments Local Ethics Committee (HRU-HADYEK) dated 09/02/2022 and numbered 2022/001.

RESULTS

The biochemical and hormonal mean values in the amniotic fluid of the study groups are presented in Table 1. Accordingly, the mean Na (103.54±0.19-103.66±0.23), glucose (3.50±0.13-7.07±0.18), K (8.28±0.20-8.49±0.10), Cl (66.31±0.34-66.63) of single and twin ewes ±0.33), Ca (3.47±0.11-1.35±0.57), P (3.94±0.97-3.62±0.12), total protein (1.27±0.86-1.35±0.76), ALT (10.30±0.18-10.67±0.17), AST (14.17±0.21-14.56±0.21), urea (79.82±0.47-79.20±0.54), creatinine (11.58±0.78-11.31±0.13) and cortisol (4.15±0.77-8.16±0.16) values were recorded. Glucose and cortisol levels were significantly higher in twin pregnancies compared to singleton pregnancies ($p<0.05$). Calcium level was significantly lower in sheep carrying twin pregnancies than in sheep carrying singleton pregnancies ($p<0.05$). There was no difference between the groups in the levels of Na, K, Cl, P, total protein, ALT, AST, urea, and creatinine in the amniotic fluid ($p>0.05$).

Table 1. The biochemical and hormonal mean values in the amniotic fluid of the study groups.

Amniotic fluid parameters	Single pregnancy		Twin pregnancy		*p
	Mean	SEM	Mean	SEM	
Na (mmol/L x 10)	103.54	0.19	103.66	0.23	0.672
Glucose (mg/ dL)	3.50	0.13	7.07	0.18	0.001
K (mmol/L)	8.28	0.20	8.49	0.10	0.382
Cl (mmol/ L x 10)	66.31	0.34	66.63	0.33	0.502
Ca / mg/ dL)	3.47	0.11	1.35	0.57	0.001
P (mmol/L)	3.94	0.97	3.62	0.12	0.63
Total protein (g/dLx0,1)	1.27	0.86	1.35	0.76	0.508
ALT(IU/L)	10.30	0.18	10.67	0.17	0.164
AST (IU/L)	14.17	0.21	14.56	0.21	0.214
Urea (mg/ dL)	79.82	0.47	79.20	0.54	0.396
Creatinine(mg/dL)	11.58	0.78	11.31	0.13	0.98
Cortisol(µg/dL)	4.15	0.77	8.16	0.16	0.001

*****Significance levels according to independent t-test results. Sodium (Na), potassium (K), chlorine (Cl), calcium (Ca), phosphorus (P), Alanine aminotransferase (ALT), Aspartate Aminotransferase (AST).

DISCUSSION

It is known that some changes occur in the developing fetus and mother during pregnancy (Narelle, 2017). More amniotic fluid is secreted in twin pregnancy for a suitable environment in developing fetuses (Hill et al. 2000). The large area created by twin fetuses also increases the amount of secreted fluid (Ippolito, et al., 2014). The fetus needs protein, carbohydrates, vitamins and minerals to maintain its healthy development throughout pregnancy (Alaçam, 1994; Arthur, Noakes & Peorsan. 1992; Küplülü, 1990). Glucose is the primary energy source for the fetus. In healthy pregnancies, fetal gluconeogenesis is very limited

and the fetus is dependent on maternal circulating glucose (Kalhan & Parimi, 2000). It has been reported that glucose concentration in amniotic fluid of twin pregnant goats is higher than that of single pregnant goats (Hay, 2006). In the present study, in accordance with the literature, it was observed that the amniotic fluid glucose concentration of twin pregnant Awassi sheep was higher than that of singleton pregnant sheep. It was thought that the reason for this might be the need for glucose due to the increased number of offspring.

However, the glucose requirement is parallel to the number of fetuses in the uterus, and glucose is secreted from the fetus into the amniotic fluid (Hay, 2006). With maternal stress, corticotropin-releasing hormone (CRH) is produced from the hypothalamus.

This hormone causes the activation of the fetal Hypothalamus-Pituitary-Adrenal (HPA) axis and the release of fetal cortisol in the amniotic fluid (Challis, Matthews, Gibb & Lye. 2000; Mbegbu, et al., 2021), in their study on Red Sokoto goats, reported that amniotic fluid cortisol concentration was higher in twin pregnant goats compared to the singleton pregnant group. In the

present study, it was higher in twin pregnancies in accordance with the literature. The reason for this may represent the cumulative response of the two developing fetuses in twin pregnancies to the increased stress due to the decreased intrauterine volume (Herman, et al., 2016). In addition, the increase in twin pregnancies; It may also be caused by the fact that cortisol is necessary for the normal development of fetal respiratory organs, liver and kidneys (Wood & Keller-Wood, 2016). Most biological processes that require energy expenditure, such as muscle contraction, blood coagulation, and bone formation, directly require calcium, thereby reducing the amount of calcium in the amniotic fluid (Mbegbu, et al., 2021; Suttle, 2010), in their study on Red Sokoto goats, reported that amniotic fluid calcium level was lower in twin pregnancies compared to singleton pregnancies. In the present study, in accordance with the literature, amniotic fluid calcium level was found to be lower in twin pregnant ewes. The decrease in amniotic fluid calcium concentrations observed in twin pregnant ewes compared to singleton pregnant ewes may be related to increased calcium use/uptake by twin fetuses during the bone formation process (Jo, et al., 2015).

In our study, no difference was found in amniotic fluid sodium, potassium, phosphorus and chlorine concentrations. It is consistent with previous studies that there were no changes in potassium, sodium, phosphorus and chlorine (Banan Khojasteh, Khadjeh, Ranjbar & Salehi 2011; Mbegbu et al., 2021). The reason why there is no difference in these minerals; It was thought that it may not have been affected by the number of offspring because the ration was sufficient for the offspring during pregnancy or because these minerals were required in very small amounts for the offspring. In the presented study, no difference was observed between twin and singleton pregnancies in urea, creatinine, AST, ALT and total protein concentrations in amniotic fluid. In previous studies, it was stated that AST, ALT and total protein levels were not affected by pregnancy and the number of developing fetuses (Carter, 1990; Pasciu et al., 2019). Since kidney and liver markers are generally related to aging and inflammation, it was thought that the relevant parameters in healthy pregnancies might not be affected by the number of fetuses. The lack of difference between the total protein values in the study is similar to the study in goats (Mbegbu et al., 2021). It has been reported that the similarity in total protein levels may be due to the fact that proteins are not filtered into fetal urine during excretion from fetal nephrons, and thus are insignificant from the number of developing fetuses (Hay, 2006).

CONCLUSION

As a result, the care-feeding conditions that will be arranged by taking into account the differences in amniotic fluid calcium, glucose and cortisol levels in twin and singleton pregnancy will contribute to the continuation and termination of the pregnancy without any problems for the mother and the offspring.

Conflict of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Author Contributions

Plan, design: TA; **Material, methods and data collection:** TA, ÖY; **Data analysis and comments:** TA, ÖY; **Writing and corrections:** TA, ÖY.

REFERENCES

Alaçam E. Gebelik Fizyolojisi. In: Alaçam E (Editör), Evcil Hayvanlarda Reprodüksiyon Suni Tohumlama, Doğum ve İnfertilite, Birinci Baskı, Konya: Dizgi evi 1994, 127-137.

Arthur G.H., Noakes D.E & Peorsan H. (1992). Infertility in The Cow: General Considerations, Anatomical, Functional and Management Causes. Veterinary Reproduction & Obstetrics (Theriogenology). London: ELBS with Bailliere Tindall.

Assis Neto A.C., Santos C.C.,Pereira F.T.V & Miglino MA. (2009). Initial Development of Bovine Placentation (Bos indicus) from the Point of View of the Allantois and Amnion. *Anatomia, Histologia, Embryologia*, 38, 341-347. https://doi.org/10.1111/j.1439-0264.2009.00949.x

Assis Neto A.C., Pereira F.T.V., Santos T.C., Ambrosio C.E., Leiser R & Miglino A. (2010). Morpho-physical Recording of Bovine Conceptus (*Bos indicus*) and Placenta from Days 20 to 70 of Pregnancy. *Reproduction in Domestic Animals*, 45, 760-772. https://doi.org/10.1111/j.1439-0531.2009.01345.x

Banan Khojasteh S.M., Khadjeh G.H., Ranjbar R & Salehi M. (2011). Studies on biochemical constituents of goat allantoic fluid during different stages of gestation. *Egyptian Journal of Sheep & Goat Sciences*, 6, 1-5.

Briese, V., Kunkel, S., Plath, C., Wutzke, K. D. & Plesse, R. (1999). Sialic acid, steroids and proteohormones in maternal, cord and retroplacental blood. *Zeitschrift fur Geburtshilfe und Neonatologie*, 203, 63-68.

Carter J. (1990). Liver function in normal pregnancy. *Australian and New Zealand Journal of Obstetrics and Gynaecology*, 30, 296-302. https://doi.org/10.1111/j.1479-828X.1990.tb02014.x

Cuckle H. (2014). Prenatal screening using maternal markers. *Journal of Clinical Medicin*e, 3, 504-520. https://doi.org/10.3390/jcm3020504.

Challis J.R.G., Matthews S.G., Gibb W & Lye S.J. (2000). Endocrine and paracrine regulation of birth at term and preterm. *Endocrinology Review*, 21, 514-550. https://doi.org/10.1210/edrv.21.5.0407.

Essawi W.M., Mostafa D.I.A. & El Shorbagy A.I.A. (2020). Comparison between biochemical analysis of cattle amniotic fluid and maternal serum components during pregnancy. *World Veterinary Journal*, 10, 67-73. https://doi.org/10.36380/scil.2020

Hay W.W. (2006). Placental-fetal glucose exchange and fetal glucose metabolism. Transactions of the *American Clinical and Climatological Association*, 117, 321-340.

Herman J.P., McKlveen J.M., Ghosal S., Kopp B., Wulsin A., Makinson R., Scheimann J. & Myers B. (2016). Regulation of the hypothalamic-pituitary-adrenocortical stress response. *Comprehensive Physiology*, 6, 603-621. https://doi.org/10.1002/cphy.c150015

Hill L.M., Krohn M, Lazebnik N., Tush B., Boyles D & Ursiny J.J. (2000). The amniotic fluid index in normal twin pregnancies. *American Journal of Obstetrics and Gynecology*, 182, 950-954. https://doi.org/10.1016/S0002-9378(00)70352-8

Ippolito D.L., Bergstrom .JE., Lutgendorf M.A., Flood-Nichols S.K & Magann E.F. (2014). A systematic review of amniotic fluid assessments in twin pregnancies. *Journal of Ultrasound in Medicine*, 33, 1353-1364. https://doi.org/10.7863/ultra.33.8.1353

Jo B.W., Shim Y.J., Choi J.H., Kim J.S., Lee H.J & Kim H.S. (2015). Formula fed twin infants with recurrent hypocalcemic seizures with vitamin D deficient rickets and hyperphosphatemia. *Annals of Pediatric Endocrinology & Metabolism*, 20, 102-105. https://doi.org/10.6065/apem.2015.20.2.102

Kalhan S. & Parimi P. (2000). Gluconeogenesis in the fetus and neonate. *Seminars in Perinatology*, 24(2), 94-106**.** https://doi.org/10.1053/sp.2000.6360

Küplülü Ş. Plasentasyon ve Gebelik Süreci in: Alaçam E (Editör). Theriogenology, Evcil Hayvanlarda Reprodüksiyon Suni Tohumlama, Obstetrik ve İnfertilite, Birinci Baskı, Ankara: Nurol Matbaacılık 1990: 97-107.

Lain, K. Y. & Catalano, P. M. (2007). Metabolic changes in pregnancy. *Clinical Obstetrics & Gynecology*, 50, 938-948. https://doi.org/10.1097/GRF.0b013e31815a5494

Mackie F.L, Whittle R, Morris R.K., Hyett J., Riley R.D & Kilby M.D. (2019). First-trimester ultrasound measurements and maternal serum biomarkers as prognostic factors in monochorionic twins: a cohort study. *Diagnostic and Prognostic Research*, 3, 9. https://doi.org/10.1186/s41512-019-0054-9

Moghaddam, G. H., & Olfati, A. (2012). Metabolic profiles in crossbreed ewes in late pregnancy. In Proceedings of the 15th AAAP Animal Science Congress, pp. 26-30.

Mbegbu E.C., Anya K.O., Oguejiofor C.F., Ugwuanyi H.E & Ohamarike I.O. (2021). Placenta morphological and amniotic fluid biochemical changes associated with mid-gestation single and twin pregnancies in red sokoto goats. International *Journal of Veterinary Science*, 10(1), 8-12. https://doi.org/10.47278/journal.ijvs/2020.014

Narelle H. (2017). Biochemical changes in pregnancy-what should a clinician know? *Journal of Gynecology and Women's Health,* 4, 555626.002. https://doi.org/10.19080/JGWH.2017.04.555626

Pasciu V., Baralla E., Nieddu M., Succu S., Porcu C., Leoni G.G., Sechi P, Bomboi GC & Berlinguer F. (2019). Commercial human kits' applicability for the determination of biochemical parameters in sheep plasma. *The Journal of Veterinary Medical Science*, 81, 294-297. https://doi.org/10.1292/jvms.18-0356

Pfarrer C., Ebert B., Miglino M.A., Klisch K. & Leiser R. (2001). The threedimensional feto-maternal vascular interrelationship during early bovine placental development: a scanning electron

microscopical study. *Journal of Anatomy*, 198, 591-602.

Prestes N.C., Chalhoub M., Lopes M.D. & Takahira R.K. (2001). Amniocentesis and biochemical evaluation of amniotic fluid in ewes at 70, 100 and 145 days of pregnancy. *Small Ruminant Research*, 39(3), 277-281. https://doi.org/10.1016/S0921-4488(00)00202-9

Rici R.E.G., Facciotti P.R., Maria D.A., Fernandes V.M., Ambrosio &Miglino M.A. (2011). Evaluation of the contribution of the placentomal fusion during gestation in cattle. *Animal Reproduction Science*, 126, 143-150. https://doi.org/10.1016/j.anireprosci.2011.06.004

Robert S.J. (1986) Veterinary obstetrics and genital disease (Theriogenelogy), 3rd edn. Edwards Brothers, Woodstock. pp 41-49.

Suttle N.F. (2010). The mineral nutrition of livestock. 4th ed. CABI International, Wallingford Suttle, MPG Books Group: London, UK, 2010; p. 565.

Schlafer D.H., Fisher P.J. & Davies C.J. (2000). The bovine placenta before and after birth: placental development and function in health and disease. *Animal Reproduction Science*, 60-61, 145-160. https://doi.org/10.1016/S0378-4320(00)00132-9

Shaw S.S., Bollini S, Nader K.A., Gastadello A., Mehta V. & Filppi E. (2011). Autologous transplantation of amniotic fluid-derived mesenchymal stem cells into sheep fetuses. *Cell Transplantation*, 20(7), 1015-1031. https://doi.org/10.3727/096368910X543402

Underwood, M. A., Gilbert, W. M. & Sherman, M. P. (2005). Amniotic fluid: Not just fetal urine anymore. *Journal of Perinatology*, 25, 341-348. https://doi.org/ 10.1038/sj.jp.7211290.

Wood C.E. & Keller-Wood M. (2016). The critical importance of the fetal hypothalamus-pituitary-adrenal axis. *F1000 Research*, 5, 115. https://doi.org/10.12688/f1000research.7224.1