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Dottorato in Scienze Veterinarie e dell'Allevamento

NON INVASIVE STUDY ON CANINE PERINATOLOGY

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To myself and to the little girl I was

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CHAPTER 1

Abstract

ABSTRACT

In order to improve knowledge about perinatal physiology in dogs, and in the attempt to provide some potential diagnostic/prognostic markers for a better management of diseased and less viable newborn puppies, the PhD project was focused on two main topics: a) the definition of some fetal fluids characteristics under normal condition; b) the investigations of suitability of hair and nail single collection for the retrospective analysis of dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS), involved in the fetal final maturation and neonatal adaptation. In relation to the first topic a first study was aimed to measure the fetal fluids cortisol concentrations in puppies at birth, and to assess the possible effect of cortisol on newborn survival at 24 hours of age, and the effect of some neonatal or maternal parameters on fetal fluids cortisol concentrations. A significant higher cortisol concentration in the allantoic than in the amniotic fluid was found, but with significant high positive correlation between amniotic and allantoic cortisol concentrations. Significant higher amniotic cortisol concentrations were found in puppies dead at 24 hours, as well a significant effect of the litter on fetal fluids cortisol concentrations. The second study investigated the concentrations of uric acid, lactate, glucose and creatinine in amniotic fluid of small sized purebred newborn dogs born by elective cesarean section at term of normal pregnancies, in relation to newborn outcome and the possible effect played by maternal parity and newborn gender on uric acid, glucose, lactate and creatinine concentrations. When the statistical analysis was performed on fetal fluids belonging to normal puppies no significant difference on uric acid concentration were found in relation to maternal parity or newborn gender. Regarding amniotic glucose concentration a significant influence of newborn gender, but not of maternal parity, was found. Amniotic lactate concentration was higher in multiparous in comparison to primiparous bitches. Regarding creatinine no significant differences were found in relation to maternal parity or newborn gender. The third study investigated the biochemical

composition of fetal fluids at term pregnancy in dogs. A comparison between fetal fluids characteristics of normal and pathologic puppies was done, but, the statistical analysis did not show significant results, due to the small number of pathologic puppies. When the statistical analysis was applied to the normal puppies, differences between the two fluids were found for many parameters, suggesting a different source and mechanism of production and accumulation of the two fluids in dogs. The study showed also the possible influence of breed body size and of maternal parity and newborn gender on some parameters. Further studies are needed in order to better investigate possible differences between fetal fluids belonging to normal and to pathological puppies, and therefore to detect potential markers of fetal/neonatal diseases or for a quick identification of newborns at risk, that need special surveillance and cares, immediately after birth. In relation to the second topic the present thesis highlighted that DHEA, recognized to be produced by the adrenals of offspring, is higher in the hair of premature as compared to puppies 1-30 days old, although not different from stillborn puppies, suggesting the possible production of this hormone by the fetus itself in the last period of intrauterine development. Although the hair is suitable for perinatal DHEA analysis in dead puppies, the hair necessary for the analysis still limits the use of this matrix for in vivo studies in newborn puppies. Therefore, the measurement of DHEA and of its transport form DHEAS, also in the nails of newborn puppies was assessed. The results showed that nails were suitable for DHEA and DHEAS measurement, allowing the use of this matrix for serial studies on alive newborn puppies. DHEA and DHEAS concentrations were significantly different in the overall concentration in nails of newborn puppies, with a high correlation between the concentrations of DHEA and DHEAS; DHEAS and not DHEA concentrations were significantly higher in small size breeds in comparison with large size breeds, while DHEAS was higher in premature puppies when compared to puppies born-dead or dead between 11 and 20 days of age. When the usefulness of these non-invasive matrices for the study of the dog

perinatology was considered, the nails resulted more suitable in comparison to the hairs. In fact, in the perspective of using these matrices in alive newborn puppies, at present the relatively large amount of hair necessary for the analysis prevent its use on alive newborns.

RIASSUNTO

Al fine di migliorare le conoscenze riguardo la fisiologia perinatale nel cane e nel tentativo di fornire dei potenziali markers diagnostici e/o prognostici per la gestione dei neonati poco vitali o patologici, il progetto di dottorato si è concentrato su due aspetti principali: a) la definizione di alcune caratteristiche dei fluidi fetali nel cane in condizioni di normalità; b) il possibile uso di pelo e unghie come matrici per lo studio retrospettivo sulla concentrazione di deidroepiandrosterone (DHEA) e deidroepiandrosterone solfato (DHEAS), coinvolti nelle fasi finali della maturazione fetale e nell'adattamento neonatale. Per quanto riguarda il primo argomento un primo studio ha indagato la concentrazione di cortisolo nei fluidi fetali del cane alla nascita, indagando il possibile effetto del cortisolo sulla sopravvivenza dei neonati a 24 ore di età, indagando inoltre l'effetto di alcuni parametri materni e fetali sulla concentrazione di cortisolo nei fluidi fetali. Lo studio ha dimostrato una concentrazione maggiore di cortisolo nel fluido allantoideo rispetto al fluido amniotico, con una correlazione positiva nelle concentrazioni tra i due fluidi, è stato riscontrato infine un effetto della cucciolata sulle concentrazioni del cortisolo nei fluidi amniotico e allantoideo. La concentrazione di cortisolo nel fluido amniotico dei cuccioli morti nelle 24 ore dopo la nascita è risultata maggiore rispetto al fluido amniotico dei neonati normali. Un secondo studio ha indagato le concentrazioni di acido urico, lattato, glucosio e creatinina nel fluido amniotico appartenente a cuccioli di taglia piccola nati da taglio cesareo elettivo al termine di gravidanze normali, valutando la possibile influenza della parità materna e del sesso dei neonati sulla concentrazione di questi metaboliti, così come sull'effetto di questi metaboliti sull'outcome neonatale. Da questo studio non sono emerse differenze significative sulla concentrazione di acido urico e creatinina in relazione a parità materna e sesso dei neonati. Per quanto riguarda il glucosio è stata riscontrata un'influenza del sesso dei neonati ma non della parità materna sulla concentrazione nel liquido amniotico. La concentrazione del lattato nel fluido amniotico è risultata

statisticamente più elevata nelle cagne multipare rispetto alle primipare. Un terzo studio ha indagato la composizione biochimica dei fluidi fetali del cane a termine di gravidanza. È stata effettuata una comparazione tra i fluidi di soggetti normali e patologici ma l'analisi statistica non è stata in grado di evidenziare differenze significative tra i due gruppi, probabilmente anche per la scarsa numerosità dei soggetti patologici rispetto ai sani. Per quanto riguarda le possibili differenze tra i fluidi nei soggetti sani, sono state evidenziate differenze significative nelle concentrazioni di alcuni dei parametri analizzati, suggerendo un diverso meccanismo di produzione e accumulo per i due fluidi nel cane. Lo studio ha rivelato inoltre l'influenza di taglia materna e parità, così come del sesso dei neonati, su alcuni dei parametri indagati. Altri studi sono necessari per meglio indagare le possibili differenze nella composizione dei fluidi fetali tra soggetti sani e patologici al fine di individuare possibili markers per la rapida individuazione dei soggetti patologici che richiedono una pronta assistenza nelle prime ore di vita. In relazione al secondo aspetto indagato è emerso che il DHEA, prodotto dai surreni fetali, presenta una concentrazione maggiore nel pelo dei neonati prematuri rispetto ai neonati morti tra 1 e 30 giorni di vita, concentrazione che risulta tuttavia simile a quella dei neonati morti nelle prime 24 ore di vita, questi risultati suggeriscono una possibile produzione di questo ormone da parte del feto stesso durante l'ultima fase di sviluppo intrauterino. Sebbene il pelo si sia rivelato una matrice utile per gli studi sulle concentrazioni del DHEA nei neonati, tuttavia il suo uso in vivo è limitato dal quantitativo richiesto per le analisi; è stata quindi indagata la concentrazione del DHEA e della sua forma di trasporto, DHEAS, anche nelle unghie dei cuccioli neonati. I risultati di questo studio hanno rivelato che le unghie sono una matrice ottimale per la determinazione di DHEA e DHEAS e che questa matrice potrebbe essere usata efficacemente anche per studi seriali in vivo. Le concentrazioni di DHEA e DHEAS nelle unghie sono risultate significativamente differenti ma fortemente e positivamente correlate. La concentrazione di DHEAS e non di DHEA è risultata

statisticamente maggiore nei cani di tagli piccola rispetto ai soggetti di taglia grande, inoltre la concentrazione di DHEAS è risultata maggiore nei cuccioli prematuri rispetto ai nati morti o ai cuccioli morti tra 11 e 20 giorni di vita.

CHAPTER 2

General Introduction and Aim

GENERAL INTRODUCTION AND AIM

BRIEF INTRODUCTION TO CANINE PERINATOLOGY

In mammals the success of the reproductive process is represented by the birth, at the physiologic term of pregnancy, of alive and viable offspring. However, the last intrauterine fetal stage of development and the neonatal period are the most challenging phases for the mammals offspring. In fact, during this periods, the fetus and the newborn undergoes to several changes necessary for the extrauterine life. Therefore, the perinatal period, that represents the time more closely around the process of birth, is considered the most critical for the offspring.

Perinatology deserved interests from several decades in the horse species and in large animals species, but at a lesser extent in the dog.

In the last years, however, the interest in canine neonatology, aimed to improve the survival of puppies after birth and to the correct management of newborn puppies, is increasing.

In fact, in dogs, perinatal mortality, represented by the number of born dead puppies plus the newborns dying during the first week of age (Tønnessen et al, 2012), is known to be relatively high (Indrebø et al, 2007), reaching values up to 30%, with an important economic impact for breeders. The majority of puppies loss (>50%) occurs during the process of whelping and in the first 24 hours of age (Gill, 2001; Indrebø et al, 2007).

The causes for puppies mortality during the perinatal period are complex and related to several factors concerning the puppy itself (low birth weight, congenital malformations), the mother (lack of milk, scarce maternal attitude), the process of birth (prolonged labor, dystocia), or the presence

of infectious agents (Gill, 2001; Davidson, 2003; Indrebø et al, 2007; Munnich, 2008; Tønnessen et al, 2012).

Puppies mortality can be reduced by a proper assistance at birth, and during the early neonatal period (Davidson , 2003). However, despite the huge scientific interest on dog reproduction, knowledge about canine perinatology are scarce, and, consequently, also neonatal assistance is remarkable lower as compared to humans (Lùcio et al, 2009; Silva et al, 2009).

Recently, the interest in canine perinatology focused on the study of intrauterine and neonatal physiology, by non invasive methods of investigation, is increasing. In fact, in canine neonates the possibility of using some methods of investigation, such as blood sampling, is limited by ethical and technical reasons. As reported by Münnich and Küchenmeister (2014) only the 10% of blood volume can be taken for investigations (blood volume in a neonate = 75 ml/kg bw) without risks for the puppy. According to these premises, some studies aimed to indeep canine perinatology focused in example on fetal fluids characteristics investigations under normal and pathological conditions (Meloni et al, 2014; Dall'ara et al 2015) and, more recently, on the use of hairs and nails (Veronesi et al, 2015) as new matrices for non invasive study in canine perinatology.

AIM OF THE PhD PROJECT

In order to improve knowledge about perinatal physiology in dogs, and in the attempt to provide some potential diagnostic or prognostic markers for a better management of diseased and less viable newborn puppies, the PhD project was focused on two main topics:

- The definition of some canine fetal fluids characteristics under normal conditions for a possible comparison with pathological conditions.
- The use of hairs and nails as a new matrices for non invasive hormonal and metabolic perinatal investigations.

CHAPTER 3

Definition of some canine fetal fluids characteristics under normal conditions for a possible comparison with pathologic conditions

DEFINITION OF SOME CANINE FETAL FLUIDS CHARACTERISTICS UNDER NORMAL CONDITIONS FOR A POSSIBLE COMPARISON WITH PATHOLOGIC CONDITIONS

THE PLACENTA IN DOGS

One of the most important events during embryonic development is the extra-embryonic membranes formation, essentials for the metabolic, gaseous and hormonal exchanges (Fresno et al, 2012).

The placenta is a transient organ that provides endocrine control and is necessary for the metabolic interchange between the conceptus and the dam (Chucri et al, 2010). According to the number of tissue layers between fetal and maternal blood, placenta can be classified as epitheliochorial, syndesmochorial, endotheliochorial and hemochorial. Dogs and cats have an endotheliochorial placentation characterized by a complete erosion of the endometrial epithelium and the underlying interstitium, with the exposure of maternal capillaries to the epithelial cells of the chorion (Aralla et al, 2013). According to the surface where feto-maternal exchanges take place in relation to the surface of the chorionic sac the placenta in dogs and cats is classified as zonary, in fact in these species the placenta shows an intimate interdigitating contact zone forming a belt around the chorionic sac (Furukawa et al, 2014).

In dogs and cats, at the margin of the placenta a lateral hematoma develops, it contains uteroverdin (Noakes et al, 2001, 2009; Verstegen-Onclin e Verstegen, 2008). Placental activities are aimed at ensuring nutritional and gaseous exchanges between mother and fetus without contact between maternal and fetal blood, performing digestive, respiratory, metabolic, endocrine and excretive functions, allowing the passage of oxygen and nutritional principles from the mother

to the fetus and catabolites from the fetus to the mother. The placenta also protects the fetus not only against traumas (fetal fluids) but also against pathogens or toxic substances.

The development of fetal-membranes in dogs and cats can be summarized as following, according to Miglino and co-workers (2006):

- 20 days of pregnancy: the yolk-sac dimension is prominent compared to the embryo and fully vascularised from the vases of umbilical cord. At this stage non-vascularised amnios fully coats the embryo and the internal surface of chorion is also non-vascularised;
- 24 days of pregnancy: fetal-membranes are easy to recognize. The yolk-sac is shaped as a reversed “T” whose ends are red for the vascularisation. The non-vascularised amnios fully coats the embryo and contains amniotic semi-transparent fluid. The allantoid is filled with a light yellow fluid that can be seen through the allantochorion, characterized by the presence of small vases. In its central zone the allantoid is in contact with the zonate placenta that forms in uterus;
- 25-30 days of pregnancy: placenta starts to show the branches of umbelical vases providing sprinkling of yolk-sac and zonate placenta. Small vases from allanthoid provide the wetting of the amnios.
- 45 days of pregnancy: the allantoid dimensions grow remarkably and it becomes fully vascularised, the amnios stays only small vascularised;
- 53 days of pregnancy: the fetus is nearly completely developed in the amnios that is completely coated by the allantoid. Macroscopically the allantoid shows a thick vascular net converging in the placenta.

FETAL FLUIDS STUDIES IN HUMANS AND OTHER DOMESTIC ANIMAL SPECIES

Fetuses are soaked in amniotic fluid originated by secretions of respiratory tract, oral cavity, gastro-intestinal tract and fetal skin before keratinization to which in the last gestation phase are added fetal urine reversed through urethra after its canalization (Fresno et al, 2012). Amniotic fluid provides to mechanical protection of the fetus and supply nutritive and molecules essential for its growth (Fresno et al, 2012). It was observed that the biochemical composition of amniotic fluid in the first phases of pregnancy is really similar to the composition of extracellular fluid of fetus in human and sheep (Fresno et al, 2012).

In mammals, during the first phases of pregnancy, the most important mechanisms of allantoid fluid storage are trans-membrane transportation and extra-embryonal membranes secreting activity; in the last phase allantoids fluid derives mainly from the developing kidneys and its composition becomes really similar to fetal urine (Fresno et al, 2012).

In mammals fetal fluids quantity varies according to pregnancy phase; in the first half of pregnancy amniotic fluid prevails then tends to decrease while allantoids fluid grows fast until half pregnancy, then decreases until birth (Noakeset al, 2009).

Fetal fluids in humans are relatively poor of organic and inorganic substances with a specific gravity slightly above water specific gravity. Allantoids fluid has a pH between 6.5-7.5 and contains uric acid, salts, urea, allantoine and glucose in traces. In amniotic fluid were found chlorates, carbonates, calcium, potassium and sodium sulfates, urea, creatine, creatinine, carbohydrates, lipids, proteins, enzymes, variable quantities of mucine, estrogens and prostaglandins. Amniotic fluid, wetting fetus surface, may contain hair, epithelial cells, skin desquamations and meconium (Underwood et al, 2005).

In human medicine the composition of amniotic fluid and the possible relation between amniotic fluid composition and the presence of pathologies in the fetus was widely investigated. Amniotic fluid contains water, peptides, proteins, lipids, carbohydrates, electrolytes and hormones. Among these components, studies have been done on amminoacids and also on trophic mediators, such as different types of grow factors (Underwood et al, 2005). The concentration and composition of fatty acids and phospholipids was proved to be related with pulmonary maturity in the fetus (Arvidson et al, 1972).

The amniotic fluid collection performed by amniocentesis was introduced more than 50 years ago as a diagnostic procedure during the second trimester of pregnancy (Jacobson et al,1967) and is considered safe in women (Norwitz and Levy, 2013).

In veterinary medicine the biochemical composition of both amniotic and allantoic fluids have been firstly studied, with particular interest in bovine (Baetz et al, 1976) and ovine (Wales et al, 1973). The researchers evidenced that the metabolic and biochemical process that take place in the fetus lead to changes in fetal fluids volume and composition (Baetz et al, 1976; Wintour et al, 1986; Peter, 2013).

In the last years, several studies were performed in mares; in particular Zanella et al (2014) focused on the biochemical composition of fetal fluids during initial, mid and final phases of gestation. Another study investigated the lactate concentration in amniotic fluid and blood in mares and foals during the early post-partum period in order to verify the possible use of this parameter in the evaluation of foal health (Pirrone et al, 2012). In fact nowadays, researches on fetal fluids are focused on the detection of some markers that could have a diagnostic/prognostic role in some gestational or neonatal pathologies (Pirrone et al, 2012; Canisso et al, 2015).

FETAL FLUIDS STUDIES IN DOMESTIC CARNIVORES

Only in the last years the attention on fetal fluids composition in small carnivores is increasing. One recent study by Fresno et al (2012) provided some essential data about the feline fetal fluids. Based on this research the feline fetal fluids composition does not represent the result of the simple filtration from maternal blood, in fact the fetus seems to have an active role in determining the biochemical composition of both amniotic and allantoic fluid throughout pregnancy. In cat the composition of amniotic and allantoic fluid tend to be similar, probably due to the diffusion from allantois vessels to amniotic cells and to the poor vascularisation of amnios. Also in feline species, as already reported for other animal species (Baetz et al 1976; Wintour et al, 1986; Prestes et al, 2001; Zanella et al, 2014), some variations in amniotic and allantoic fluid composition occur along gestation, as the reflex of changes in transfer and metabolic activity and differences in the contribution of fetal and placental tissues to the amniotic and allantoic compartments.

Because in dogs amniocentesis is difficult to perform due to the anatomy of pregnant animal uterus, fetal sacs, and fluids (Prestes et al, 2001) the scientific and clinical interests for fetal fluids composition was not developed until recently. In fact, Meloni et al (2014), and subsequently also Dall'ara et al (2015), reported that both amniotic and allantoic fluids can be safely collected at the time of birth, especially during caesarean section performed at term of pregnancy for the health of both mother and puppies. Cesarean section is a routine procedure in bitches at high risk of dystocia, such as brachicephalus bitches or small size bitches, and is also performed in bitches with an history of troubles at parturition. The collection of fetal fluids during surgery is safe and do not interfere with neonatal care. In the aforementioned studies, bitches were fully monitored from before the artificial insemination (AI) or the mating, throughout gestation, until parturition. To better define the best day for mating or AI, all the bitches were monitored during the estrus cycle by vaginal cytology and plasma progesterone analysis (von Heimendahl et al, 2010). Pregnancy

diagnosis was done by ultrasonography between 20 and 28 days after mating or AI, when the inner chorionic cavity allowed the estimation of parturition date (Luvoni and Gironi, 2000; Luvoni and Beccaglia, 2006); to better define the parturition date and to evaluate a second time the viability of fetuses, at 40 to 45 days of gestation another ultrasound examination was performed and the measurement of the biparietal diameter was done. At the estimated parturition day, the bitches were submitted to an elective cesarean section only if plasma progesterone was under 2 ng/ml. Fetal fluids were collected for each fetus contemporary at the extraction of the fetus at the opening of fetal sacs with a sterile syringe without the needle. Those studies allowed to improve the knowledge about some aspects of canine fetal fluids composition, under normal conditions. In fact the study from Dall'Ara et al (2015) reported the presence of Immunoglobulins G and lysozyme in fetal fluids of dogs at term of pregnancy demonstrating that amniotic and allantoic fluid play a role in the protection of the fetus/newborn also in dogs, as already reported in humans (Quan et al, 1999; Akinbi et al, 2004) and underlying the role of the placenta in the passive transfer of immunoglobulins to the fetus during gestation. Meloni et al (2014) investigated the Insulin-like Growth Factors-I (IGF-I) and Non-esterified Fatty Acids (NEFA) concentrations in dog fetal fluids at term pregnancy and demonstrated that these substances are strongly related to the breed size. Those preliminary studies evidenced the importance of improving knowledge on fetal fluids characteristics under normal and also pathological conditions suggesting a possible diagnostic or prognostic role of fetal fluids analysis for a better neonatal assistance and to reduce neonatal mortality in newborn puppies.

In a recent study, Balogh et al (2017), reported the use of semi-quantitative tests to differentiate canine fetal fluids from maternal urine on the basis of biochemical characteristics, suggesting a possible use of these tests as additional tools to recognize parturition in term pregnant bitches if there is a clear-yellowish vulvar discharge in clinical setting. In that study, fetal fluids from the

fetus nearest to the site of hysterectomy and maternal urines were collected during elective cesarean section from 26 bitches. Fetal fluids and urines were than analyzed and, in particular, Specific Gravity was analyzed with a refractometer, pH, leukocytes, nitrite, protein, glucose, ketones, urobilinogen, bilirubin and erythrocyte/hemoglobin content were evaluated with the Combur-Test® strips (Roche Diagnostics AG, Rotkreuz, Switzerland). Also the concentration of calcium and magnesium were detected using a semi-quantitative test. Finally the Placental alpha Microglobulin-1 (PAMG-1) protein concentration was investigated using the AmniSure test. From a practical point of view, the results of thiat study were particularly interesting becausethey demonstrated the possible use of semi-quantitative tests under field conditions for the identification of the fetal membranes rupture in dogs.

CHAPTER 4

References

REFERENCES

Akinbi HT, Narendran V, Pass AK, Markart P, Hoath SP. (2004) Host defense proteins in vernix caseosa and amniotic fluid. Am J Obstet Gynecol 2004; 191(6):2090-6.

Aralla M, Groppetti D, Caldarini L, Cremonesi F, Arrighi S. (2013) Morphological evaluation of the placenta and fetal membranes during canine pregnancy from early implantation to term. Res Vet Sci 95(1), 15-22.

Arvidson G, Ekelund H, Astedt B. (1972) Phospholipid composition of human amniotic fluid during gestation and at term. Acta Obstet Gynecol Scand. 1972;51(1):71-5

Baetz AL, Hubbert WT, Gram CK. (1976) Changes of biochemical constituents in bovine foetal fluids with gestational age. Am j vet res, 37: 1047-1052.

Balogh O, Roch M, Keller S, Michel E, Reichler IM. (2017) The use of semi-quantitative tests at Cesarean section delivery for the differentiation of canine fetalfluids from maternal urine on the basis of biochemical characteristics. Theriogenology. 2017 Jan 15;88:174-182. doi: 10.1016/j.theriogenology.2016.09.024. Epub 2016 Sep 21.

Canisso IF, Ball BA, Scoggin KE, Squires EL, Williams NM, Troedsson MH. (2015) Alpha-fetoprotein is present in the fetal fluids and is increased in plasma of mares with experimentally induced ascending placentitis. Anim Reprod Sci, doi: 10.1016/j.anireprosci.2014.12.019

Chucri TM, Monteiro JM, Lima AR, Salvadori MLB, Kfoury Junior JR, Miglino MA. (2010) A review of immune transfer by the placenta. J Reprod Immunol 87, 14-20.

Dall'Ara P, Meloni T, Rota A, Servida F, Filipe J, Veronesi MC. (2015) Immunoglobulins G and lysozyme concentrations in canine fetal fluids at term of pregnancy. Theriogenology 2015; 83:766-71.

Davidson AP. (2003) Approaches to Reducing Neonatal Mortality in Dogs. In Recent Advances in Small Animal Reproduction, Concannon PW, England G, Verstegen J, Linde-Forsberg C. (Eds.) Publisher: International Veterinary Information Service (www.ivis.org), Ithaca, New York, USA.

Fresno L, Rodriguez-Gil JE, RigauT, Pastor J, Rivera del Alamo MM. (2012). Modulation of the biochemical composition of amniotic and allantoic fluids as a control mechanism of feline foetal development. Placenta 33 (2012) 522e527.

Furukawa S, Kuroda Y, Sugiyama A. (2014) A comparison of the histological structure of the placenta in experimental animals. J Toxicol Pathol. 2014 Apr;27(1):11-8.

Gill MA. (2001) Perinatal and late neonatal mortality in the dog. University of Sydney (2001) PhD Thesis. http://hdl.handle.net/2123/4137.

Indrebø A, Trangerud C, Moe L. (2007) Canine neonatal mortality in four large breeds. Acta Vet Scand 49(Suppl I), S2.

Jacobson CB, Barter RH. (1967) Intrauterine diagnosis and management of genetic defects. Am J Obstet Gynecol. 1967 Nov 15;99(6):796-807.

Lúcio CF, Silva LCG, Rodrigues JA, Veiga GAL, Vannucchi CI. (2009) Peripartum haemodynamic status of bitches with normal birth or dystocia. Reprod Domest Anim 2009; 44:133-6.

Luvoni GC, Beccaglia M. (2006) The prediction of parturition date in canine pregnancy. Reprod Dom Anim 41, 27-32.

Luvoni GC, Grioni A. (2000) Determination of gestational age in medium and small size bitches using ultrasonographic fetal measurements. J Small Anim Pract 41, 292-4.

Meloni T, Comin A, Rota A, Peric T, Contri A, Veronesi MC. (2014) IGF-I and NEFA concentrations in fetal fluids of term pregnancy dogs. Theriogenology 2014; 81:1307-11.

Miglino MA, Ambrósio CE, dos Santos Martins D, Valverde Wenceslau C, Pfarrer C, Leiser R. (2006) The carnivore pregnancy: the development of the embryo and fetal membranes. Theriogenology, 66, 1699-1702.

Münnich A and Küchenmeister U. (2014) Causes, Diagnosis and Therapy of Common Diseases in Neonatal Puppies in the First Days of Life: Cornerstones of Practical Approach. Reprod Dom Anim 49 (Suppl. 2), 64–74 (2014); doi: 10.1111/rda.12329

Münnich A. (2008) The pathological newborn in small animals: the neonate is not a small adult. Vet Res Commun. 2008 Sep;32 Suppl 1:S81-5. doi: 10.1007/s11259-008-9096-0.

Noakes D E, Parkinson TJ, England GCW. (2009) Veterinary reproduction and obstetrics. Ninth edition.

Noakes DE, Parkinson TJ, England GCW. (2001) Arthur's ostetricia e riproduzione veterinaria. Ottava edizione.

Norwitz ER, Levy B. (2013) Noninvasive Prenatal Testing: The Future Is Now. Rev Obstet Gynecol 6(2), 48-62.

Peter AT. (2013) Bovine placenta: A review on morphology, components, and defects from terminology and clinical perspectives. Theriogenology 80(7), 693-705.

Pirrone A, Mariella J, Gentilini F, Castagnetti C. (2012) Amniotic fluid and blood lactate concentrations in mares and foals in the early postpartum period. Theriogenology 78(6), 1182-1189.

Prestes NC, Chalhoub MC, Lopes MD, Takahira RK. (2001) Amniocentesis and biochemical evaluation of amniotic fluid in ewes at 70, 100 and 145 days of pregnancy. Small Rumin Res. 2001 Mar;39(3):277-281.

Quan CP, Forestier F, Bouvet JP. (1999) Immunoglobulins of the human amniotic fluid. Am J Reprod Immunol 1999; 42:219-225.

Silva LCG, Lúcio CF, Veiga GAL, Rodrigues JA, Vannucchi CI. (2009) Neonatal clinical evaluation, blood gas and radiographic assessment after normal birth, vaginal dystocia or caesarean section in dogs. Reprod Domest Anim 2009; 44:160-3.

Tønnessen R, Borge KS, Nødtvedt A, Indrebø A. (2012) Canine perinatal mortality: a cohort study of 224 breeds. Theriogenology 2012; 77:1788-801.

Underwood MA, Gilbert WM, Sherman MP. (2005) Amniotic fluid: not just fetal urine anymore. J Perinatol 25, 341-348.

Veronesi MC, Comin A, Meloni T, Faustini M, Rota A, Prandi A. (2015) Coat and claws as new matrices for noninvasive long-term cortisol assessment in dogs from birth up to 30 days of age. Theriogenology 2015; 84:791-6.

Verstegen-Onclin K, Verstegen J. (2008) Endocrinology of pregnancy in the dog: a review. Theriogenology, 70, 291-299.

von Heimendahl A, England GCW. (2010) Determining breeding status. In: England G, von Heimendahl A, editors. BSAVA Manual of Canine and Feline Reproduction and Neonatology, 2nd ed, BVSAVA, Gloucester; 2010, p. 44-50.

Wales RG, Muedoch RN. (1973). Changes in the composition of sheep foetal fluids during early pregnancy. J reprod fertil, 33:197-205.

Wintour EM, Laurence BM, Lingwood BE. (1986) Anatomy, physiology and pathology of the amniotic and allantoic compartments in the sheep and cow. Aust Vet J 63, 216-221.

Zanella LF, Takahira RK, Melo e Oña CM, Oña Magalhães LC, Prestes NC. (2014) Biochemical profile of amniotic and allantoic fluid during different gestational phases in mares. J Equine Vet Sci 34(3), 203-206.

CHAPTER 5

Aim of the first step of the PhD project

AIM OF THE FIRST STEP OF THE PhD PROJECT

In human medicine the investigation of fetal fluids characteristics is one of the tools actually used to investigate fetal and neonatal well-being. Also in veterinary medicine this topic was documented in all domestic animals, but not in dogs until recently, when the interest about the investigation of fetal fluids characteristics started to increase. In fact, only in the last years some aspects of canine fetal fluids were investigated, but to the authors knowledge the characteristics of amniotic and allantoic fluids during different stages of normal pregnancy and also the possible changes in fetal fluids under pathological conditions are lacking in dogs. In order to provide knowledge for a better neonatal assistance in dogs, and in the attempt to provide some diagnostic and prognostic markers for the early recognition of less viable or pathologic newborns puppies, the PhD project focused on the analysis of fetal fluids as a non invasive method for the study of neonatology in dogs.

The present thesis represents only a starting point in the investigation of fetal fluids characteristics under normal conditions. Specifically, the cortisol concentration in amniotic and allantoic fluid of normal, viable and well-developed puppies, born by normal pregnancies, was investigated as a useful tool for the detection of hypothalamic-pituitary-adrenal axis activity of fetuses during the last phase of gestation. Furthermore the biochemical composition of fetal fluids collected at term of pregnancy was assessed in order to better define fetal fluids characteristics under normal conditions, with the objective to define a starting point for future investigations also under pathological conditions.

Uric acid, glucose, lactate and creatinine are considered markers of fetal metabolism in humans, and therefore the concentration of these parameters in canine amniotic fluid were also

investigated, because of their possible role in fetal and neonatal well-being and development, also in dogs.

Finally, the possible use of urinary dipsticks for the investigation of fetal fluids “rough” biochemical composition immediately after birth was defined on a group of normal, healthy and viable newborns, for the future comparison with diseased or less viable puppies.

Further researches are necessary to identify possible correlations between fetal fluids characteristics and maternal or fetal/neonatal diseases in general, and to identify possible markers of fetal and neonatal well-being disturbances in order to improve neonatal assistance in dogs.

CHAPTER 6

Cortisol fetal fluids concentrations in term pregnancy small-sized purebred dogs and preliminary relation to newborn first 24 hours survival

CORTISOL FETAL FLUIDS CONCENTRATIONS IN TERM PREGNANCY SMALL-SIZED PUREBRED DOGS AND PRELIMINARY RELATION TO NEWBORN FIRST 24 HOURS SURVIVAL

The last stages of pregnancy and the neonatal period represent two stressful stages, for mammals fetus and newborn. In dogs, natimortality and neonatal loss reaches values up to 30% with a notable economic impact for breeders (Indrebø et al, 2007; Tønnessen et al, 2012). In dogs several factors are responsible for this high perinatal mortality rate including the puppy itself (low birth weight, congenital malformations), the mother (lack of milk, scarce maternal attitude), the process of birth (prolonged labor, dystocia), or the presence of infectious agents (Gill, 2001; Davidson et al, 2003; Indrebø, 2007; Munnich et al, 2008; Tønnessen et al, 2012). More than the 50% of neonatal losses occurs during the process of whelping and in the first 24 hours after birth (Gill, 2001; Indrebø et al, 2007). Puppies mortality can be reduced by a proper assistance at birth, and during the early neonatal period (Davidson et al, 2003). However knowledge about canine perinatology are scarce and consequently the assistance given to puppies at whelping and during the neonatal period are remarkably lower as compared to humans (Lùcio et al, 2009, Silva et al, 2009). Therefore the assistance provided to puppies is often empirical, so that to reduce the impact of canine neonatal death, the improvement of knowledge about fetal development physiology in the last intrauterine stages of gestation, as well as of the process of birth and neonatal adaptation, is necessary.

In animal species, birth is triggered by the activation of the fetal hypothalamic-pituitary-adrenal (HPA) axis; in fact, an enhance in fetal plasma glucocorticoids was reported during late gestation (Challis et al., 2000; Challis et al., 2001; Schwartz and McMillen, 2001). The timing of HPA axis activation in carnivores is still unknown (Vannucchi et al, 2012). Cortisol, a corticosteroid hormone, is synthesized by the adrenal glands under the regulation of the anterior pituitary adrenocorticotropic hormone (ACTH) which is stimulated by the corticotrophin-releasing hormone

(CRH) that is secreted by the hypothalamic paraventricular nucleus. CRH and arginine-vasopressin (AVP) work as the principal modulators of HPA axis (Mastorakos and Illas, 2003).

Glucocorticoids are involved in fetal maturation, regulation of immune response, and several physiological changes correlated with gestation (Gonzales et al, 1986; Whittle et al, 2001; Myatt and Sun, 2010). Furthermore, fetal cortisol seems to have a critical role in the initiation of parturition (Challis et al, 2000; Whittle et al, 2001; Jenkin and Young, 2004).

Studies in sheep demonstrated that the maturing HPA axis causes the release of increasing amount of cortisol towards the end of pregnancy. At placental level, cortisol induces the expression of enzymes responsible for the synthesis of estrogen and prostaglandin, which may stimulate uterine contractions (Anderson et al, 1975; Flint et al, 1978; Ma et al, 1999).

Multiple endocrine factors play a role in the transition to extrauterine life. In particular, some days before parturition, the physiological increase of endogenous cortisol concentrations is of outmost importance for fetal final maturation. In fact, in humans, high cortisol levels in fetal circulation are correlated to the lung maturation (Bonanno and Wapner, 2009; Vannucchi et al, 2012). Several events occur for the lung maturation such as changes in interstitial tissue components, cellular maturation and differentiation, surfactant protein and phospholipid production, and regulation of the pulmonary fluids metabolism (Bolt et al, 2001; Vannucchi et al, 2012). In human medicine, antenatal corticosteroids administration to pregnant women is a routine treatment to artificially induce pulmonary fetal maturation and significantly decrease the incidence of respiratory distress syndrome in premature newborns (Peltoniemi et al, 2007; Vannucchi et al, 2012). Also in ovine fetuses it was documented that the antenatal steroids exposure improves the adaptation after birth by accelerating lung maturation (Houfflin-Debarge et al, 2005).

The role of cortisol in the regulation of surfactant synthesis in fetal lungs was widely described in both humans and animals (Gonzales et al., 1986; Mendelson et al., 1986). During the intrauterine

development, the surfactant is synthesized by type II alveolar cells and intermittently discharged into the amniotic fluid (Van Golde et al., 1988; Pryhuber et al., 1991).

Hypothalamic-pituitary-adrenal axis development is associated with a gradual increase of ACTH and adrenal corticosteroids in the fetal circulation during the last days of pregnancy (Challis et al., 2000; Challis et al., 2001). Although the specific mechanisms initiating parturition can be different among animal species, glucocorticoids were proposed as the factors synchronizing the fetal maturation with the trigger of labor in nearly all species investigated (Jenkin and Young, 2004).

The activity of HPA and cortisol levels in fetuses and newborns were previously investigated in humans by fetal blood or cord blood analysis (Fisk et al, 2001; Kaiantie et al, 2004), but these methods are now limited by the invasiveness of sampling. In fact blood sampling for perinatal investigations are not advisable from both ethical and technical prospective, in humans and also in other species. Cortisol levels were also investigated in amniotic fluid in humans, and its increase in the last weeks of pregnancy associated to lung maturity (de Fencl and Tulchinsky, 1975). In animal species, cortisol concentrations were investigated in the tammar wallaby (Ingram et al, 1999), the sheep (Challis et al, 1981) and, more recently, the cat (Fresno et al, 2012). In dogs, the collection of fetal fluids at birth was proved to be safe for both, mother and puppies, without interfering with neonatal care, and useful for several investigations as reported by Meloni et al (2014). In fact fetal fluids are easily collectable during cesarean section, frequently performed in bitches at high risk of dystocia, or in bitches with previous history of trouble at parturition (Meloni et al, 2014).

REFERENCES

Bolt RJ, van Weissenbruch MM, Lafeber HN, Delemarre-van de Waal HA. (2001) Glucocorticoids and lung development in the fetus and preterm infant. Pediatr Pulmonol. 2001 Jul;32(1):76-91.

Bonanno C, Wapner RJ. (2009) Antenatal corticosteroid treatment: what's happened since Drs Liggins and Howie? Am J Obstet Gynecol. 2009 Apr;200(4):448-57. doi: 0.1016/j.ajog.2008.12.011.

Challis J, Sloboda D, Matthews S, Holloway A, Alfaidy N, Howe D, Fraser M, Newnham J. (2000) Fetal hypothalamic-pituitary adrenal (HPA) development and activation as a determinant of the timing of birth, and of postnatal disease. Endocr Res 2000; 26: 489-504.

Challis JR, Patrick JE, Cross J, Workewych J, Manchester E, Power S. (1981) Short-term fluctuations in the concentration of cortisol and progesterone in fetal plasma, maternal plasma, and amniotic and allantoic fluids from sheep during late pregnancy. Can J Physiol Pharmacol 1981; 59:261-7.

Challis JR, Sloboda D, Matthews SG, Holloway A, Alfaidy N, Patel FA, Whittle W, Fraser M, Moss TJ, Newnham J. (2001) The fetal placental hypothalamic-pituitary-adrenal (HPA) axis, parturition and post natal health. Mol Cell Endocrinol 2001; 185:135-44.

Davidson AP. (2003) Approaches to Reducing Neonatal Mortality in Dogs. In Recent Advances in Small Animal Reproduction, Concannon PW, England G, Verstegen J, Linde-Forsberg C. (Eds.) Publisher: International Veterinary Information Service (www.ivis.org), Ithaca, New York, USA.

de Fencl M, Tulchinsky D. (1975) Total cortisol in amniotic fluid and fetal lung maturation. N Engl J Med 1975; 292:133-6.

Fisk NM, Gitau R, Teixeira JM, Giannakoulopoulos X, Cameron AD, Glover VA. (2001) Effect of direct fetal opioid analgesia on fetal hormonal and hemodynamic stress response to intrauterine needling. Anesthesiology 2001; 95:828-35.

Gonzales LW, Ballard PL, Ertsey R, Williams MC. (1986) Glucocorticoids and thyroid hormones stimulate biochemical and morphological differentiation of human fetal lung in organ culture. J Clin Endocrinol Metab. 1986 Apr;62(4):678-91.

Houfflin-Debarge V, Deruelle P, Jaillard S, Magnenant E, Riou Y, Devisme L, Puech F, Storme L. (2008) Effects of antenatal glucocorticoids on circulatory adaptation at birth in the ovine fetus. Biol Neonate. 2005;88(2):73-8. Epub 2005 Mar 21.

Indrebø A, Trangerud C, Moe L. (2007) Canine neonatal mortality in four large breeds. Acta Vet Scand 49(Suppl I), S2.

Ingram JN, Shaw G, Renfree MB. (1999) Cortisol in fetal fluids and the fetal adrenal at parturition in the tammar wallaby (Macropus eugenii). Biol Reprod 1999; 60:651-5.

Jenkin G, Young IR. (2004) Mechanisms responsible for parturition; the use of experimental models. Anim Reprod Sci. 2004 Jul;82-83:567-81. Review.

Kajantie E, Raivio T, Jänne OA, Hovi P, Dunkel L, Andersson S. (2004) Circulating glucocorticoid bioactivity in the preterm newborn after antenatal betamethasone treatment. J Clin Endocrinol Metab. 2004 Aug;89(8):3999-4003.

Lúcio CF, Silva LCG, Rodrigues JA, Veiga GAL, Vannucchi CI. (2009) Peripartum haemodynamic status of bitches with normal birth or dystocia. Reprod Domest Anim 2009; 44:133-6.

Mastorakos G, Illas I. (2003) Maternal and fetal hypothalamic-pituitary-adrenal axis during pregnancy and parturition. Ann N Y Acad Sci 997, 136-49.

Mendelson CR, Chen C, Boggaram V, Zacharias C, Snyder JM. (1986) Regulation of the synthesis of the major surfactant apoprotein in fetal rabbit lung tissue. J Biol Chem. 1986 Jul 25;261(21):9938-43.

Münnich A. (2008) The pathological newborn in small animals: the neonate is not a small adult. Vet Res Commun. 2008 Sep;32 Suppl 1:S81-5. doi: 10.1007/s11259-008-9096-0.

Myatt L, Sun K. (2010) Role of fetal membranes in signaling of fetal maturation and parturition. Int J Dev Biol. 2010;54(2-3):545-53. doi: 10.1387/ijdb.082771lm. Review.

Peltoniemi OM, Kari MA, Tammela O, Lehtonen L, Marttila R, Halmesmäki E, Jouppila P, Hallman M. (2007) Repeat Antenatal Betamethasone Study Group. Randomized trial of a single repeat dose of prenatal betamethasone treatment in imminent preterm birth. Pediatrics. 2007 Feb;119(2):290-8.

Pryhuber GS, Hull WM, Fink I, McMahan MJ, Whitsett JA. (1991) Ontogeny of surfactant proteins A and B in human amniotic fluid as indices of fetal lung maturity. Pediatr Res. 1991 Dec;30(6):597-605.

Schwartz J, McMillen IC. (2001) Fetal hypothalamic-pituitary-adrenal axis on the road of parturition. Clin Exp Pharmacol Physiol 28(1-2), 108-12.

Silva LCG, Lúcio CF, Veiga GAL, Rodrigues JA, Vannucchi CI. (2009) Neonatal clinical evaluation, blood gas and radiographic assessment after normal birth, vaginal dystocia or caesarean section in dogs. Reprod Domest Anim 2009; 44:160-3.

Snyder JM, Johnston JM, Mendelson CR. (1981) Differentiation of type II cells of human fetal lung in vitro.Cell Tissue Res. 1981;220(1):17-25.

Snyder JM, Kwun JE, O'Brien JA, Rosenfeld CR, Odom MJ. (1988) The concentration of the 35-kDa surfactant apoprotein in amniotic fluid from normal and diabetic pregnancies. Pediatr Res. 1988 Dec;24(6):728-34.

Tønnessen R, Borge KS, Nødtvedt A, Indrebø A. (2012) Canine perinatal mortality: a cohort study of 224 breeds. Theriogenology 2012; 77:1788-801.

Van Golde LM, Batenburg JJ, Robertson B. (1988) The pulmonary surfactant system: biochemical aspects and functional significance. Physiol Rev. 1988 Apr;68(2):374-455. Review.

Vannucchi CI, Silva LC, Lúcio CF, Regazzi FM, Veiga GA, Angrimani DS. (2012) Prenatal and neonatal adaptations with a focus on the respiratory system. Reprod Domest Anim 47 Suppl 6, 177-81.

Whittle WL, Patel FA, Alfaidy N, Holloway AC, Fraser M, Gyomorey S, Lye SJ, Gibb W, Challis JR. (2001) Glucocorticoid regulation of human and ovine parturition: the relationship between fetal hypothalamic-pituitaryadrenal axis activation and intrauterine prostaglandin production. Biol Reprod 64, 1019-1032.

CHAPTER 7

Cortisol fetal fluids concentrations in term pregnancy small-sized purebred dogs and preliminary relation to newborn first 24 hours survival

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Cortisol fetal fluid concentrations in term pregnancy of small-sized purebred dogs and its preliminary relation to first 24 hours survival of newborns

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ABSTRACT

Along the perinatal period, in mammals, cortisol (C) plays a pivotal role in the final intrauterine fetal maturation and in the early neonatal adaptation. Because of the scarce knowledge about canine perinatology, the present study was aimed to assess the C concentrations in amniotic and allantoic fluids collected, without invasiveness, from small-sized, purebred newborn puppies born by elective cesarean section, at term of pregnancy. Possible correlations between fetal fluid C concentrations and maternal parity, litter size, birth weight, Apgar score, were evaluated. In addition, the possible effect of fetal fluid C concentrations on newborn survival at 24 hours of age, and the effect of the litter or the newborn gender on fetal fluid C concentrations were also assessed. The results, obtained from 50 born alive, normal-weight puppies, without gross physical malformation, showed that C concentration was higher in allantoic than in amniotic fluid ($P < 0.01$), even if a strong positive correlation between the two fluids C concentration was found ($P < 0.0001$; $R = 0.83$). Neither amniotic nor allantoic C concentrations were correlated to maternal parity, litter size, birth weight, and Apgar score. Interestingly, higher amniotic ($P < 0.05$), but not allantoic, C concentrations were found in puppies not surviving at 24 hours after birth. Therefore, it could be suggested that this parameter may be useful for the recognition, at birth, of puppies needing special surveillance during the first day of age. A significant ($P < 0.001$) effect of the litter in both amniotic and allantoic C concentrations was found. In conclusion, the present results showed that in small-sized purebred puppies, born at term by elective cesarean section, the exact fetal, maternal, or placental source contributing to fetal fluid C concentrations remains to be clarified. From a clinical perspective, however, the evaluation of amniotic C concentration at birth seems useful for the detection of puppies that need special surveillance during the first 24 hours of age, and should be coupled to the early newborn evaluation by Apgar score. However, the small total number of newborns, and especially of the dead puppies enrolled in the present study, suggests that further, more-focused investigations on a large number of subjects are needed before the method could be considered for application in the clinical practices.

1. Introduction

In mammals, the full success of the reproductive process is achieved by the birth, at the physiologic term of pregnancy, of alive and viable offspring, able to survive and to

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become adults. The process of birth is considered a challenge for the transition of a fetus to a neonate, and also the first hours after birth could hamper the neonatal ability to adapt to the new extrauterine life. Therefore, the perinatal period, which pertains to the period immediately before and after birth, is considered the most critical for the offspring.

In dogs, perinatal losses, represented by the number of born-dead puppies plus the newborns dying during the first week of age [1], are surprisingly high [2], reaching value up to 30%, with a notable economic impact for breeders. The high canine perinatal loss rate can be addressed to several factors including the bitch, the process of birth, the puppy itself, or the environment [2]. The majority or puppy losses (>50%) occurs during the process of whelping and in the first 24 hours of age [3]. Although the control of such a high perinatal loss rate could be complex, puppies' mortality must be reduced by a correct management of the whole reproductive process, and especially by a proper assistance at birth, and during the early neonatal period [4]. However, despite the huge scientific interest on dog reproduction, knowledge about canine perinatology are scarce, and, consequently, also neonatal assistance is remarkably lower as compared to humans [5,6]. Hence, the assistance provided to the puppies at the time of birth is often empirical [5,6], both during normal parturition and also when dystocia occurs.

Dystocia was reported to account about 5% of whelpings in dogs [7], but it occurs more frequently in some breeds, such as the brachycephalic breeds [8] and particularly in English bulldogs [9]. Miniature and small-sized purebred bitches were also reported to be at high incidence (59.4%) of dystocia [10].

Toward term of pregnancy and immediately after birth, the hypothalamic-pituitary-adrenal axis (HPA) is a key system regulating several physiologic processes, including the multiorgans fetal final maturation [11], the response to stress [12], and after birth, when the newborn experiences a series of physiologic and metabolic changes necessary for survival, the HPA is crucial for the neonatal adaptation to the new extrauterine life [13,14]. In response to the adrenocorticotrophic hormone, the adrenals produce cortisol (C), involved in several antenatal and postnatal processes. The activity of fetal and neonatal HPA axes and C levels were previously investigated in humans by fetal blood [15] or cord blood analysis [16]. However, these methods are currently limited by the invasiveness of sampling. In fact, from ethical and technical perspectives, perinatal studies based on newborn blood samples analysis are not advisable, in humans and also in other species. In the dog, the collection of fetal fluids at birth was proved to be safe for the puppy, without interfering with neonatal care, and useful for several investigations [17,18]. Fetal fluids are easily collectable during cesarean section, a procedure performed frequently as elective surgery in many bitches belonging to breeds at high risk for dystocia, or with previous history of trouble at parturition, for the health of both the mothers and puppies [18].

In humans, amniotic C concentrations increase abruptly in the last weeks of pregnancy [19,20] and are associated to fetal lung maturity [19]. About the origin of amniotic fluid C, de Fencl et al. [21] reported that unconjugated corticosteroids may be produced from various sources and subjected to interconversion, and the fetal urines are considered as the main source of corticosteroid conjugates in humans' amniotic fluid. The fetal origin of amniotic C was also previously suggested by Murphy et al. [22], which found a better correlation between amniotic fluid C and umbilical cord C in comparison to that of amniotic fluid C and maternal serum C.

The allantoic C concentrations received lower interest in comparison to their amniotic counterparts and were mainly investigated in some animal species such as the sheep [23] and in the tammar wallaby [24]. In the latter, the allantoic C increased toward term of pregnancy with a mean C concentration of about 40 ng/mL. More recently, in cats [25], reported that C allantoic concentrations reached the highest at term, with concentrations of about 55 ng/mL.

Some parameters, such as the neonatal gender, can affect fetal fluid C concentrations. For example, Torday et al. [26] found higher amniotic C concentrations in females in comparison to male human fetuses.

The chance of survival in newborn puppies also depend on body weight at birth [3,27] and by the index of viability, evaluable by the Apgar score measurement [8,28–30]. The role of the litter size [1,3,8], and of maternal parity [1,3], were also considered as factors involved in the neonatal mortality in dogs.

To the authors' knowledge, the possible relation between fetal fluid C concentrations and the likelihood to survive after birth was never investigated in small-sized purebred dogs.

To provide knowledge for a better neonatal assistance to newborn dogs, and because of the scarce knowledge about C fetal fluid concentrations in dogs, the present study was aimed to: (1) measure the amniotic and allantoic C concentrations, and to verify possible differences and correlations between the C concentrations in the two types of fluids in small-sized purebred newborn puppies born by elective cesarean section, at term of pregnancy; (2) to assess the possible effect of fetal fluid C concentrations on newborn survival at 24 hours of age, and the effect of the litter and newborn gender on fetal fluid C concentrations; and (3) to assess the possible correlation between fetal fluid C concentrations and maternal parity, litter size, birth weight, and Apgar score.

2. Materials and methods

2.1. Animals

The study enrolled 19 purebred, 2- to 6-year old, 1 to 5 parity, small-sized bitches (body weight < 10 kg), belonging to several breeds—11 Chihuahua, four Maltese, one miniature Bull terrier, one Spitz, one Pug, and one Dachshund. All the bitches belong to breeds at high risk of dystocia, or single bitches showed previous troubles at parturition. For these reasons, all the bitches underwent an elective cesarean section for the health of mothers and puppies, at term of pregnancy [18]. The bitches were healthy, regularly vaccinated and dewormed, and were clinically monitored from the time of breeding to the end of

pregnancy [18]. The day of cesarean section was defined on the basis of ovulation day estimation coupled to fetal ultrasonographic measurement of both the inner chorionic cavity and biparietal diameter, and by blood progesterone concentrations monitoring, as reported by Meloni et al. [18].

2.2. Cesarean section and newborn management

Before surgery, all the owners signed an informed consent not only for surgery, but specifically for the fetal fluids collection and use for research purposes.

Cesarean section was performed using always the same protocol for anesthesia, aimed to minimize the effect of drugs on newborns, as previously described by Meloni et al. [18]. Neonatal care and fetal fluid collection were always performed by two different operators: one for neonatal care and one for fluids collection. The amniotic and allantoic fluids were aseptically collected from each puppy, immediately centrifuged at $\times$1000g for 10 minutes, and the supernatant stored at -20 °C, until analysis by RIA. A total of 46 amniotic and 41 allantoic fluid samples were collected.

Within 5 minutes after birth, all the newborns were evaluated for viability by Apgar score [28], gender, absence of gross physical malformations, and weighed before nursing. Litter size was also recorded.

2.3. Fetal fluid C analysis

Amniotic and allantoic C concentrations were analyzed by a solid-phase microtiter RIA procedure. In brief, a 96-well microtiter plate (OptiPlate, PerkinElmer Life Science, Boston, MA, USA) was coated with goat antirabbit γ-globulin serum, diluted 1:1000 in 0.15-mM sodium acetate buffer, pH 9, and incubated overnight at 4 °C. The plate was washed twice with RIA buffer, pH 7.4, and incubated overnight at 4 °C with 200 μL of the anticortisol serum diluted 1:12000. The rabbit anti-C antibody used was obtained from Biogenesis (Poole, UK). The crossreactivities of this antibody with other steroids are as follows: C 100%, corticosterone 1.8% and aldosterone less than 0.02%. After washing the plate with RIA buffer, standards (5–300 pg/well), the quality control extract, the test extracts, and the tracer (Hydrocortisone (Cortisol, [1,2,6,7-3H (N)]), Perkin–Elmer Life Sciences, Boston, MA, USA) were added. The plate was incubated overnight at 4 °C. Bound hormone was separated from free hormone by decanting the extract and washing the wells in RIA buffer. After the addition of 200-μL scintillation cocktail, the plate was counted on a beta-counter (Top-Count, PerkinElmer Life Sciences, Boston, MA, USA). The intra-assay and interassay coefficients of variation were 4.8% and 8.7%, respectively. The sensitivity of the assay was defined as the dose of hormone at 90% binding (B/B0) and was 2.94 pg/well. To determine the parallelism between C standards and endogenous C in fetal fluids, samples containing high concentrations of endogenous C were serially diluted in PBS 0.05 M, pH 7.5. The relationship between the fetal fluid C and standard C curve determined through linear regression was linear—the correlation coefficient r was 0.99 and 0.98, the model was given by the equation $y = 0.9490x + 0.279$ and $y = 0.9586x + 0.153$ for amniotic and allantoic fluids, respectively.

2.4. Statistical analysis

Possible differences in C concentrations between the two types of fluids were statistically evaluated by Wilcoxon test, whereas the correlation between C concentration in the two fluids and the correlation between fetal fluids C concentrations and maternal parity, litter size, birth weight, and Apgar score were assessed by the Spearman correlation test. The possible effect of fetal fluid C concentrations on newborn survival at 24 hours of age, as well as the possible effect played by newborn gender on fetal fluid C concentrations, were assessed by the Mann–Whitney test, whereas the possible effect of the litter was assessed by Kruskal–Wallis test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Clinical findings

The 19 small-sized purebred bitches, 12 multiparous (2–5 parity) and seven primiparous, gave birth to 50 born alive puppies, 28 males and 22 females, without gross physical malformations. Litter size ranged between one and six (mean ± SD: 3.6 ± 1.34) and birth weight ranged between 70 and 250 g (mean ± SD: 159.9 ± 46.02 g). According to Apgar score, as reported by Veronesi et al. [28], 47 (94%) puppies were normally viable (Apgar score ≥7), while one (2%) was moderately depressed (Apgar score between 4 and 6), and two (4%) were severely depressed (Apgar score <4). At 24 hours after birth, four of 50 puppies (8%) died; the two severely depressed newborns, the one moderately depressed (despite the initial positive response to the neonatal resuscitation, the three puppies died in the first hours after birth), and one puppy with normal viability at birth, found dead after 12 hours of birth. Those four puppies belonged to different litters and breeds. For all of the dead puppies, abnormal maternal behaviors or illness was excluded. In all the dead puppies, necropsy did not show evidence of gross malformations, trauma, or infections.

3.2. Statistical findings

The statistical analysis showed a significant difference in C median concentrations between the two fluids, with higher median C concentrations in the allantoic in comparison to the amniotic fluid (Table 1). However, the Spearman correlation test showed a significantly high ($P < 0.0001$), positive correlation ($R = 0.83$) between amniotic and allantoic C concentrations.

A significant ($P < 0.05$) effect of amniotic C concentration and newborn survival at 24 hours of age was found. In fact, higher amniotic C concentrations were found in puppies dead at 24 hours after birth, in comparison to puppies alive at 24 hours after birth. Even if apparently higher median C concentrations were detected in allantoic fluids of puppies dead at 24 hours of age in comparison to

Table 1
Concentrations of C (ng/mL) in amniotic and allantoic fluids (values expressed as median, first, and third quartiles).

Fetal fluids	Cortisol concentrations (ng/mL)		
	Median	First quartile	Third quartile
Amniotic fluid (N = 46)	3.7[a]	2.3	11.1
Allantoic fluid (N = 41)	6.7[b]	4.0	11.6

[a, b] $P < 0.01$ between median values.

puppies alive at 24 hours of age, the statistical analysis failed to detect significant differences (Table 2).

A significant ($P < 0.001$) effect of the litter was found in both amniotic and allantoic C concentrations.

No significant correlations between amniotic or allantoic C concentrations and maternal parity, litter size, birth weight, and Apgar score were found.

4. Discussion

In the aim to provide knowledge about canine fetal fluid C concentrations for better assistance of puppies at birth, the present study investigated the C concentrations in amniotic and allantoic fluids collected by small-sized purebred puppies, born by elective cesarean section performed at term of pregnancy. The possible differences in C concentrations between the two types of fluids, the correlation between amniotic and allantoic C concentrations, the correlation between the fetal fluid C concentrations and maternal parity, litter size, birth weight, Apgar score, as well as the effects of fetal fluid C concentrations on newborn survival at 24 hours of age, and the effect of the litter and of the newborn gender on fetal fluid C concentrations were assessed.

The results showed that significantly ($P < 0.01$) higher C concentrations in allantoic in comparison to amniotic fluid, and a strong ($P < 0.0001$) positive correlation between C concentrations between the two fluids were found. Also in sheeps, C allantoic concentrations were reported to be higher than in the amniotic fluid [23]. Although in humans, de Fencl et al. [21], reported that the main source of amniotic C are fetal urines, the exact origin of amniotic C in dogs is difficult to define. Canine amniotic C concentrations could result by a possible concurrence of maternal counterpart and the fetal production, or even by a placental involvement in the final amniotic fluid C accumulation. On the other hand, the canine allantoic fluid more likely results from the fetal metabolism because of the large presence of fetal urines, so that C allantoic concentrations could be likely addressed to the fetus itself. However, in swine, allantoic fluid seems to originate by the maternal compartment, and serve as a nutrient reservoir and not only for fetal waste [31]. The finding of a significative, positive correlation between amniotic and allantoic C concentrations, seems to reinforce the hypothesis of a major fetal concurrence to C levels in both fluids, in dogs, similarly to what reported in the cat [25]. On the other hand, the significant effect of litter on both amniotic and allantoic C concentrations, explaining the wide among-litter variation, seems to support a major role played by the maternal compartment or the placental fetomaternal interface on both fetal fluid C concentrations.

Interestingly, significantly higher ($P < 0.05$) amniotic C concentrations, but not C allantoic concentrations, were found in puppies dead at 24 hours after birth in comparison to puppies alive at 24 hours of age. This result suggests that amniotic C concentrations measured at birth could be prognostic for short-term survival in newborn puppies and could be very important for a prompt detection of puppies that need special surveillance during the first 24 hours of age. Although apparently higher allantoic C concentrations were also found in puppies dead at 24 hours after birth, the statistical analysis failed to detect significant effect of allantoic C concentrations and puppies survival at 24 hours after birth, most likely because of the effect of the marked interindividual variability.

Recently, the measurement of the Apgar score at birth, proved the usefulness of this method for the prompt identification of low viable newborns, needing special immediate neonatal care or assistance, also in the dog [8,28–30]. In the present study the lack of significant correlation between fetal fluid C concentrations and Apgar score seems to suggest that the two methods should be used together for a better monitoring of puppies during the first day after birth. In the present study, in fact, among the four puppies not surviving at 24 hours after birth, three were born with low Apgar score and their viability improved with neonatal resuscitation, whereas one puppy showed high Apgar score, but was dead at 24 hours of age. Because those puppies not surviving at 24 hours after birth showed higher amniotic C concentrations than puppies alive at 24 hours of age, it is possible to hypothesize that, despite the initial positive response to resuscitation, those puppies had underlying conditions unsuitable for the complex process of neonatal adaptation, even after the initial positive response to resuscitation. In humans, amniotic C concentrations

Table 2
Amniotic and allantoic (median, first, and third quartiles) C concentrations (ng/mL) in puppies surviving or dead at 24 h of age, and in males and females.

Neonatal parameters	Cortisol concentrations (ng/mL)					
	Median value		First quartile		Third quartile	
	AM (N = 46)	AL (N = 41)	AM (N = 46)	AL (N = 41)	AM (N = 46)	AL (N = 41)
Surviving at 24 h (N = 46)	3.9[a]	6.7	2.8	3.9	11.1	11.5
Dead at 24 h (N = 4)	11.2[b]	10.6	7.2	8.4	12.2	11.9
Males (N = 28)	7.2	7.2	2.9	3.1	11.1	11.3
Females (N = 22)	3.7	6.7	2.8	4.5	11.7	14.3

[a, b] $P < 0.05$ between median values.
Abbreviations: AL, allantoic fluid; AM, amniotic fluid.

were related to lung maturation [11], so that a respiratory immaturity or impairment could be suspected also in not-surviving puppies in the present study. In fact, the initial positive response to reanimation in three of four dead puppies did not rule out that other underlying respiratory (or other) adaptational processes were impaired. However, the small number of not-surviving puppies in the present study prevents every speculation about the actual cause-effect relation between higher amniotic fluid C concentration and the puppies' death. To clarify this, further more-focused investigations are necessary and should add to the routine postmortem examination more specific investigations, such as the immunohistochemical evaluation of lung maturity.

This hypothesis seems to be confirmed also by the findings at necropsy, since no evidences of gross malformations, trauma, or infections were detected. The lack of correlation between fetal fluid C concentrations and Apgar score seems to suggest that the two parameters provide different, and complementary, tools for the newborn dog evaluation after birth and deserves further investigations. From a clinical perspective, it seems possible to suggest that the Apgar score is suitable for the soon evaluation of puppies at birth, to establish the prompt resuscitation, and to evaluate the immediate effectiveness of resuscitation, but the amniotic C measurement could detect those puppies that need special surveillance during the first 24 hours of age, independently by the Apgar score improvement after resuscitation. On further evidences of lung or respiratory immaturity or impairments as the cause of death, the special surveillance would include the hospitalization of the at-risk neonates and, the oxygenation status monitoring (and implementation, when needed) of the newborn, and/or the possible surfactant administration.

No significant correlations between fetal fluid C concentrations and maternal parity, litter size, and birth weight were detected, as well as no effects of neonatal gender on fetal fluid C concentrations. To the authors' knowledge, the correlation between maternal parity on C fetal fluid concentration was never investigated in both humans and domestic animals, although Tønnessen et al. [1] reported that perinatal mortality in dogs is influenced by the number of litters and Gill [3] reported maternal parity as a significant predictor of mortality in newborn puppies. In the present study, this parameter was analyzed also because Dall'Ara et al. [17] found that maternal parity affected the IgG concentrations in canine fetal fluids. According to litter size, the average 3.6 puppies/litter found in the present study, agrees with the 3.5 to 4.2 mean litter size reported for miniature and small breeds, respectively, by Borge et al. [32], and with the 3.6 for toy breeds reported by Gill [3]. Because perinatal mortality in dogs was reported to be associated to increasing litter size [1], or at the litter size extremes in toy breeds [3], it could be possible that, in the present study, no significant correlations between litter size and fetal fluid C concentrations were found because none of the litters were large or at the extremes. Birth weight in newborn dogs is considered an important prognostic factor for surviving, and newborn puppies with low body weight at birth are considered at risk for neonatal diseases and mortality [3,27,33], especially in toy breeds and small-sized breeds [3]. It should be noted that, in the present study, none of the puppy showed low body weight at birth. In fact, even if some Chihuahua puppies showed body weight as low as 70 g, to the authors experience, many normal, surviving, Chihuahua puppies, usually show birth weight of 70 g (unpublished data). The absence of puppies with low body weight at birth could, at least in part, explain the absence of significant correlations between fetal fluid C concentrations and puppies' birth weight. On the other hand, neonatal low birth weight and the causes of high C fetal fluid concentrations could act independently when affecting puppies' short-term survival. The lack of significant differences in fetal fluid C concentrations according to newborn gender is in contrast to the lower C concentrations found in the amniotic fluid of male fetuses in humans by Torday et al [26].

4.1. Conclusions

In conclusion, the results obtained in the present study showed that higher C concentrations are detectable in allantoic in comparison to amniotic fluid in small-sized purebred dogs born by elective caesarean section at term. Amniotic and allantoic C concentrations are strongly significantly positively correlated each other, suggesting at least a concurrence of the fetus itself in the fetal fluid C accumulation, even if the among-litter variation seems to suggest a maternal role in the final C fetal fluid concentrations. From a clinical perspective, the significant effect of higher amniotic C concentrations on negative newborn outcome at 24 hours of age seems to suggests that C amniotic measurement at birth, may be by portable analyzers, could be useful for the detection of puppies that need special surveillance during the first 24 hours of age, and should be coupled to the Apgar score. However, this finding was drawn from a small total number of newborns, and especially from a very little number of dead puppies; moreover, more-focused postmortem examinations of dead puppies were not performed. Thus, these results must be considered cautiously, and further, more-focused investigations on a larger number of subjects are needed before the method could be considered for application in the clinical practices.

The lack of significant correlations between fetal fluid C concentrations and maternal parity, litter size, birth weight, and especially Apgar score, and the absence of significant effect of newborn gender on fetal fluid C concentrations deserve further investigations.

References

[1] Tønnessen R, Borge KS, Nødtvedt A, Indrebø A. Canine perinatal mortality: a cohort study of 224 breeds. Theriogenology 2012;77:1788–801.
[2] Indrebø A, Trangerud C, Moe L. Canine neonatal mortality in four large breeds. Acta Vet Scand 2007;49:S2.
[3] Gill MA. Perinatal and late neonatal mortality in the dog. PhD Thesis. Sydney, Australia: University of Sydney, http://hdl.handle.net/2123/4137; 2001.
[4] Davidson AP. Approaches to reducing neonatal mortality in dogs. In: Concannon PW, England G, Verstegen J, Linde-Forsberg C, editors. Recent Advances in Small Animal Reproduction, IVIS 2003.

[5] Lúcio CF, Silva LCG, Rodrigues JA, Veiga GAL, Vannucchi CI. Peripartum haemodynamic status of bitches with normal birth or dystocia. Reprod Domest Anim 2009;44:133–6.
[6] Silva LCG, Lúcio CF, Veiga GAL, Rodrigues JA, Vannucchi CI. Neonatal clinical evaluation, blood gas and radiographic assessment after normal birth, vaginal dystocia or caesarean section in dogs. Reprod Domest Anim 2009;44:160–3.
[7] Linde-Forsberg C, Eneroth A. Abnormalities in pregnancy, parturition, and the periparturient period. In: Ettinger SJ, Feldman EC, editors. Textbook of Veterinary Internal Medicine Expert Consult. Philadelphia: WB Saunders, Co; 2000.
[8] Batista M, Moreno C, Vilar J, Golding M, Brito C, Santana M, et al. Neonatal viability evaluation by Apgar score in puppies delivered by cesarean section in two brachycephalic breeds (English and French bulldog). Anim Reprod Sci 2014;146:218–26.
[9] Wydooghe E, Berghmans E, Rijsselaere T, Van Soom A. International breeder inquiry into the reproduction of the English bulldog. Vlaams Diergeneeskundig Tijdschrift 2013;82:38–43.
[10] Münnich A, Küchenmeister U. Dystocia in numbers - evidence-based parameters for intervention in the dog: causes for dystocia and treatment recommendations. Reprod Domest Anim 2009;44: 141–7.
[11] Bolt RJ, van Weissenbruch MM, Lafeber HN, Delemarre-van de Waal HA. Glucocorticoids and lung development in the fetus and preterm infant. Pediatr Pulmonol 2001;32:76–91.
[12] Tegethoff M, Raul JS, Jamey C, Khelil MB, Ludes B, Meinlschmidt G. Dehydroepiandrosterone in nails of infants: a potential biomarker of intrauterine responses to maternal stress. Biol Psychol 2011;87: 414–20.
[13] Challis J, Sloboda D, Matthews S, Holloway A, Alfaidy N, Howe D, et al. Fetal hypothalamic-pituitary adrenal (HPA) development and activation as a determinant of the timing of birth, and of postnatal disease. Endocr Res 2000;26:489–504.
[14] Challis JR, Sloboda D, Matthews SG, Holloway A, Alfaidy N, Patel FA, et al. The fetal placental hypothalamic-pituitary-adrenal (HPA) axis, parturition and post natal health. Mol Cell Endocrinol 2001;185: 135–44.
[15] Fisk NM, Gitau R, Teixeira JM, Giannakoulopoulos X, Cameron AD, Glover VA. Effect of direct fetal opioid analgesia on fetal hormonal and hemodynamic stress response to intrauterine needling. Anesthesiology 2001;95:828–35.
[16] Kajantie E, Raivio T, Jänne OA, Hovi P, Dunkel L, Andersson S. Circulating glucocorticoid bioactivity in the preterm newborn after antenatal betamethasone treatment. J Clin Endocrinol Metab 2004; 89:3999–4003.
[17] Dall'Ara P, Meloni T, Rota A, Servida F, Filipe J, Veronesi MC. Immunoglobulins G and lysozyme concentrations in canine fetal fluids at term of pregnancy. Theriogenology 2015;83:766–71.
[18] Meloni T, Comin A, Rota A, Peric T, Contri A, Veronesi MC. IGF-I and NEFA concentrations in fetal fluids of term pregnancy dogs. Theriogenology 2014;81:1307–11.
[19] de Fencl M, Tulchinsky D. Total cortisol in amniotic fluid and fetal lung maturation. N Engl J Med 1975;292:133–6.
[20] Divers WA, Babaknia A, Hopper BR, Wilkes MM, Yen SS. Fetal lung maturation: amniotic fluid catecholamines, phospholipids, and cortisol. Am J Obstet Gynecol 1982;142:440–4.
[21] de Fencl M, Koos B, Tulchinsky D. Origin of corticosteroids in amniotic fluid. J Clin Endocrinol Metab 2009;50:431–6.
[22] Murphy BEP, Patrick J, Denton RL, Clark SJ, Marvin M, Haïdar R. Cortisol in amniotic fluid during human gestation. J Clin Endocrinol Metab 1975;40:164–7.
[23] Challis JR, Patrick JE, Cross J, Workewych J, Manchester E, Power S. Short-term fluctuations in the concentration of cortisol and progesterone in fetal plasma, maternal plasma, and amniotic and allantoic fluids from sheep during late pregnancy. Can J Physiol Pharmacol 1981;59:261–7.
[24] Ingram JN, Shaw G, Renfree MB. Cortisol in fetal fluids and the fetal adrenal at parturition in the tammar wallaby (*Macropus eugenii*). Biol Reprod 1999;60:651–5.
[25] Fresno L, Rodriguez-Gil JE, Rigau T, Pastor J, Rivera del Alam MM. Modulation of the biochemical composition of amniotic and allantoic fluids as a control mechanism of feline foetal development. Placenta 2012;33:522–7.
[26] Torday JS, Nielsen HC, de Fencl M, Avery ME. Sex differences in fetal lung maturation. Am Rev Respir Dis 1981;123:205–8.
[27] Mosier JE. The puppy from birth to six weeks. Vet Clin North Am 1978;8:79–100.
[28] Veronesi MC, Panzani S, Faustini M, Rota A. An Apgar scoring system for routine assessment of newborn puppy viability and short-term survival prognosis. Theriogenology 2009;72:401–7.
[29] Doebeli A, Michel E, Bettschart R, Hartnack S, Reichler IM. Apgar score after induction of anesthesia for canine caesarean section with alfaxalone versus propofol. Theriogenology 2013; 80:850–4.
[30] Vassalo FG, Simões CR, Sudano MJ, Prestes NC, Lopes MD, Chiacchio SB, et al. Topics in the routine assessment of newborn puppy viability. Top Companion Anim Med 2015;30: 16–21.
[31] Bazer FW, Johnson GA. Pig blastocyst-uterine interactions. Differentiation 2014;87:52–65.
[32] Borge KS, Tønnessen R, Nødtvedt A, Indrebø A. Litter size at birth in purebred dogs–a retrospective study of 224 breeds. Theriogenology 2011;75:911–9.
[33] Blunden AS. A review of the fading puppy syndrome (also known as fading puppy complex). Vet Annu 1986;26:264–9.

CHAPTER 8

Uric acid, glucose, lactate, creatinine concentrations and lactate/creatinine in the amniotic fluid and newborn outcome in small-sized purebred dogs

URIC ACID, GLUCOSE, LACTATE, CREATININE CONCENTRATIONS AND LACTATE/CREATININE IN THE AMNIOTIC FLUID AND NEWBORN OUTCOME IN SMALL-SIZED PUREBRED DOGS

In mammals fetal development and well-being are under the control of several factors, among them, the placenta and fetal fluids are responsible for the maintenance of a suitable environment for the fetus. The amniotic fluid is composed by the secretions of respiratory and gastrointestinal tract with the contribution of oral cavity and fetal skin before keratinization, and, in the last stages of pregnancy, it also contains fetal urine. The composition of amniotic fluid changes along pregnancy course, also in relation to fetal, maternal or placental contribution and could reflect the metabolism of these compartments (Underwood et al, 2005).

In humans the composition of amniotic fluid during pregnancy was widely investigated in the last fifty years, and some aspects fully elucidated. In fact, in humans the analysis of amniotic fluidprovided the detection of markers for the early recognition of fetal well-being or disturbances, as well as for maternal disturbances, allowing a more correct management of the fetus/newborn or the pregnant woman (Eguiluz et al, 1983; Bader et al, 1995).

Also in animal species the composition of fetal fluids received scientific interest, in particular in ruminants and horses, where fetal fluids were studied in normal and also in pathological conditions and markers of fetal well-being disturbances were found (Kochhar et al, 1997; Pirrone et al, 2012).

In human medicine, perinatal asphyxia is considered one of the most important and common cause of stillbirth and neonatal dead (Banupriya et al, 2008). Hypoxia leads to anaerobic metabolism with the activation of glycolysis to generate adenosine triphosphate(ATP), but only 2 molecules of ATP are produced during anaerobic conditions instead of 38 molecules produced

during aerobic conditions, the lack of ATP leads to a rise in adenosine diphosphate (ADP) and adenosine monophosphate (AMP) concentrations; these products are then catabolized to adenosine, inosine and hypoxantine. When hypoxia condition persists, hypoxantine is oxidized to xantine and uric acid (Bader et al, 1995; Chen et al, 2000; Liu et al, 2006; Pallab Basu et al, 2008; Banupriya et al, 2008).

In dogs, during the process of birth under normal conditions, a transient and short term asphyxia occurs and it lasts up to 60 minutes (Grundy et al, 2006, Vassalo et al, 2015). In dogs, in fact, the process of parturition could be very long (12 hours), in particular when dystocia occurs or when the litter-size is large (Indrebø et al, 2007; Tønnessen et al, 2012). Because of this long fetal expulsion phase, coupled to many other factors, such as the litter size, and other factors related to the mother and/or to the puppies, perinatal death in dogs can reach high percentages (30%)(Indrebø et al, 2007; Tønnessen et al, 2012). Newborn dogs in which hypoxia do not recover within 60 minutes after birth are more susceptible for neonatal disease such as septicemia or puppy fading syndrome, and are exposed to an increased risk of neonatal death (Johnston et al, 2001). Therefore, the negative effects of prolonged asphyxia at birth can results in an increase of puppies loss also after the day of birth. In fact most of the neonatal losses occurs during the process of whelping and during the first week of age reaching values up to 30%, with an economic impact for the breeders.

The knowledge of the last intrauterine fetal stages of development and the neonatal period in dogs could help to reduce this extremely high perinatal loss . In this context, the collection and the analysis of fetal fluids characteristics under normal and also pathological conditions is one of the possible tool for the non-invasive study of this crucial period.

Because of the high metabolism and the very little and short-term energetic storages of the newborn puppy, another pivotal factor that can affect neonatal survival in newborn dogs is the availability of proper energetic sources (Vassalo et al, 2015). In mammals the amniotic fluid is swallowed by the fetus during the last stages of pregnancy in order to prepare the digestive system for his future role, some of the amniotic constituents, such as carbohydrates and proteins, are digested and adsorbed by the fetus (Noaks et al, 2009). Indeed the amniotic fluid is considered as a possible source of nutrients for the developing fetus. Among the different constituents of amniotic fluid, glucose is recognized as the major metabolic fuel for the fetus, and also for the newborn immediately after birth. Studies in humans demonstrated that amniotic fluid glucose concentrations are related to the maturity of fetal kidney, in fact lower amniotic glucose levels are found with the progression of pregnancy (Spellacy et al, 1973), while low amniotic fluid glucose concentrations were found in patients with congenital defects (Stefos et al, 2003). In rats, a study reported a positive correlation between birth weight and amniotic fluid glucose concentrations (Koski and Ferguson, 1992).

Not only the glucose is considered a marker of fetal maturity, in fact also lactate and uric acid were considered indicators of fetal maturity and also of possible metabolic distress. Uric acid concentration in amniotic fluid was negatively correlated with birth weight in rats (Koski and Ferguson, 1992). In humans elevated concentrations of uric acid in maternal blood was proved to be related with preeclampsia and low birth weight (Roberts et al, 2005). In fact, in humans, a relation between uric acid concentration in amniotic fluid and the placental amino-acid uptake was found in preeclamptic pregnancies (Bainbridge et al, 2009). Moreover, in a study from Tao Gao et al (2008), uric acid concentration in amniotic fluid was negatively associated with infant birth weight and a correlation between uric acid concentration in amniotic fluid and gestational

age was found. All these studies suggested the possible use of uric acid measurement in amniotic fluid as a marker of fetal metabolism and fetal growth in human medicine.

Fetal blood or cord blood lactate has been considered a marker of fetal distress for years, in particular it has been used for detecting the presence and the severity of fetal distress because it increases rapidly in response to hypoxia (Eguiuz et al, 1983; Liu and Mantsch, 1999). In humans, lactate concentrations in amniotic fluid were described as a good marker of the uterine metabolic status during labor (Wiberg-Itzel et al, 2008; Wiberg-Itzel et al, 2011; Wiberg-Itzel et al, 2014); in fact high levels of lactate were associated with the decrease of the uterus oxygenation, prolonged labor and increased risk of adverse neonatal or maternal outcome, while normal lactate concentrations were associated with normal delivery (Wiberg-Itzel et al, 2008; Wiberg-Itzel et al, 2011; Wiberg-Itzel et al, 2014). In a recent study, Wiberg-Itzel et al (2014) found an important association between lactate levels in amniotic fluid and oxitocin administration, with an increased risk of adverse neonatal outcome in newborn infants. Some authors reported that, to better estimate fetal lacticaemia, a normalization on amniotic fluid volume by the calculation of lactate to creatinine ratio is necessary (Torrance et al, 2013).

In humans creatinine is a normal constituent of amniotic fluid and, at term of pregnancy, it is considered a marker of fetal excretional activity. Some studies in humans demonstrated that creatinine levels in amniotic fluid are related to birth weight and fetal maturity (Parmley and Miller, 1969; Doran et al, 1970). The analysis of creatinine and urea in amniotic fluid was also reported as a method to evaluate renal maturation and functionality throughout pregnancy (Oliveira et al, 2002).

REFERENCES

Bader D, Gozal D, Weinger-Abend M, Berger A, Lanir A. (1995). Neonatal urinary uric acid/creatinine [correction of creatinine] ratio as an additional marker of perinatal asphyxia. Eur J Pediatr, 154, 747-749.

Bainbridge SA, Deng JS, Roberts JM. (2009) Increased xanthine oxidase in the skin of pre-eclamptic women. Reprod Sci, 2009;16:468–78.

Banupriya C, Ratnakar, Dourerdjou P, Mondal N, Bhat Vishnu, Koner BC. (2008). Can urinary excretion rate of malondialdehyde, uric acid and protein predict the severity and impending death in perinatal asphyxia? Clin Biochem, 41, 968-973.

Basu P, Som S, Choudhuri N, Das H. (2008) Correlation between Apgar score and urinary uric acid to creatinine ratio in perinatal asphyxia. Indian J Clin Biochem. 2008 Oct;23(4):361-4. doi: 10.1007/s12291-008-0079-2. Epub 2008 Dec 20.

Chen HJ, Yau KIT, Tsai KS. (2000). Urinary uric acid/creatinine ratio as an additional marker of perinatal asphyxia. J Formos Med Assoc, 99, 771-774.

Dall'Ara P, Meloni T, Rota A, Servida F, Filipe J, Veronesi MC. (2015) Immunoglobulins G and lysozyme concentrations in canine fetal fluids at term of pregnancy. Theriogenology 2015; 83:766-71.

Doran TA, Bjerre S, Porter CJ. (1970) Creatinine, uric acid, and electrolytes in amniotic fluid. Am J Obstet Gynecol. 1970 Feb 1;106(3):325-32

Eguiluz A, López Bernal A, McPherson K, Parrilla JJ, Abad L. (1983) The use of intrapartum fetal blood lactate measurements for the early diagnosis of fetal distress. Am J Obstet Gynecol1983;147:949-54

Gao T, Zablith NR, Burns DH, Skinner CD, Koski KG. (2008) Second trimester amniotic fluid transferrin and uric acid predict infant birth outcomes. Prenat Diagn 2008;28:810-4.

Grundy SA. (2006). Clinically relevant physiology of the neonate. Vet Clin North Am: Small Animal Practice, 36, 443-459.

Indrebø A, Trangerud C, Moe L. (2007) Canine neonatal mortality in four large breeds. Acta Vet Scand 49(Suppl I), S2.

Kochhar HPS, Simran PS, Nanda AS, Kaur R. (1997) Comparative biochemical indices of fetal fluids in normal foaling and stressful delivery in Indian thoroughbred mares. Journal of Equine Veterinary Science, Volume 17, Issue 4, April 1997, Pages 206-210

Koski KG, Ferguson MA. (1992). Amniotic fluid composition responds to changes in maternal dietary carbohydrate and is related to metabolic status in term fetal rats. J Nutr, 122, 385-392.

Liu L, Zhou HY, Song JW, Nie WX, Su ZY. (2006). Measurement of urinary S100B protein levels and lactate/creatinine ratio in early detection of neonatal hypoxic-ischemic encephalopathy. World J Pediatr, 2, 270-275.

Liu KZ, Mantsch HH. (1999) Simultaneous quantitation from infrared spectra of glucose concentrations, lactate concentrations, and lecithin/sphingomyelin ratios in amniotic fluid. Am J Obstet Gynecol. 1999 Mar;180(3 Pt 1):696-702.

Meloni T, Comin A, Rota A, Peric T, Contri A, Veronesi MC. (2014) IGF-I and NEFA concentrations in fetal fluids of term pregnancy dogs. Theriogenology 2014; 81:1307-11.

Noakes D E, Parkinson TJ, England GCW. (2009) Veterinary reproduction and obstetrics. Ninth edition.

Oliveira FR, Barros EG, Magalhães JA. (2002)Biochemical profile of amniotic fluid for the assessment of fetal and renal development. Brazilian journal of Medical and Biological Research (2002) 35: 215-222

Parmley T, Miller E. (1969) Fetal maturity and amniotic fluid analysis. Am J Obstet Gynecol. 1969 Oct 1;105(3):354-62.

Pirrone A, Mariella J, Gentilini F, Castagnetti C. (2012) Amniotic fluid and blood lactate concentrations in mares and foals in the early postpartum period. Theriogenology 78(6), 1182-1189.

Roberts JM, Bodnar LM, Lain KY, Hubel CA, Markovic N, Ness RB, et al. (2005) Uric acid is as important as proteinuria in identifying fetal risk in women with gestational hypertension. Hypertension 2005;46:1263–9.

Spellacy WN, Buhi WC, Bradley B, Holsinger KK. (1973) Maternal, fetal and amniotic fluid levels of glucose, insulin and growth hormone. Obstet Gynecol. 1973 Mar;41(3):323-31.

Stefos T, Sotiriadis A, Kaponis A, Dalkalitsis N, Lolis D. (2003) Amniotic fluid glucose at the time of genetic amniocentesis: correlation with duration of pregnancy and birthweight. Eur J Obstet Gynecol Reprod Biol. 2003 Feb 10;106(2):144-7.

Tønnessen R, Borge KS, Nødtvedt A, Indrebø A. (2012) Canine perinatal mortality: a cohort study of 224 breeds. Theriogenology 2012; 77:1788-801.

Torrance HL, Pistorius L, Voorbij HA et al. (2013) Lactate to creatinine ratio in amniotic fluid: a pilot study. J Matern Fetal Neonatal Med. 2013.Vol. 26,N 7.P. 728–730.

Underwood MA, Gilbert WM, Sherman MP. (2005) Amniotic fluid: not just fetal urine anymore. J Perinatol 25, 341-348.

Vassallo FG, Simões CR, Sudano MJ, Prestes NC, Lopes MD, Chiacchio SB, Lourenco ML. (2015). Topics in the routine assessment of newborn puppy viability. Topics in Companion Animal Medicine, 30, 16-21.

Wiberg-Itzel E, Akerud H Andolf E, Hellström-Westas L, Winbladh B, Wennerholm UB. (2011) Association Between Adverse Neonatal Outcome and Lactate Concentration in Amniotic Fluid. Obstet Gyn: July 2011 - Volume 118 - Issue 1 - p 135-142

Wiberg-Itzel E, Pembe AB, Wray S, Wihlbäck AC, Darj E, Hoesli I, Åkerud H. (2014). Level of lactate in amniotic fluid and its relation to the use of oxytocin and adverse neonatal outcome. Acta Obstet Gyn Scan, 93, 80-85.

Wiberg-Itzel E, Lipponer C, Norman M, Herbst A, Prebensen D, Hansson A, Bryngelsson AL, Christoffersson M, Sennström M, Wennerholm UB, Nordström L. (2008) Determination of pH or lactate in fetal scalp blood in management of intrapartum fetal distress: ra ndomised controlled multicentre trial. BMJ. 2008 Jun 7;336(7656):1284-7. doi:10.1136/bmj.39553.406991.25. Epub 2008 May 25.

CHAPTER 9

Uric acid, glucose, lactate, creatinine concentrations and lactate/creatinine in the amniotic fluid and newborn outcome in small-sized purebred dogs

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Association of amniotic uric acid, glucose, lactate and creatinine concentrations and lactate/creatinine ratio with newborn survival in small-sized dogs – preliminary results

Amniotic uric acid, glucose, lactate and creatinine in dogs

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ABSTRACT

In the purpose to define the normal composition of canine amniotic fluid and to detect differences between surviving and not surviving newborn puppies, the present study was aimed to define the uric acid, glucose, lactate, creatinine concentrations and the lactate: creatinine ratio in amniotic

fluids collected during elective Caesarean section from small-sized purebred bitches. The possible relation between newborn survival and studied parameters, as well as the effects of maternal parity, fetal gender and Apgar score were assessed. The study enrolled 27 small-sized purebred bitches, and submitted to elective Caesarean section, at term. The amniotic fluid samples were collected, after fetal membranes opening, from each fetus, aseptically by the amniotic sac. The data, obtained from 74 amniotic samples collected from 27 bitches, showed that amniotic glucose concentration was lower ($p<0.05$) in non surviving in comparison to surviving puppies. Within the normal, surviving puppies, glucose amniotic concentration was higher ($p<0.05$) in males as compared to females newborns and lactate: creatinine ratio was significantly higher in multiparous than in primiparous bitches ($p<0.05$). The preliminary results evidenced the relevance of amniotic glucose, but not uric acid, lactate, creatinine and lactate to creatinine ratio for detecting puppies at risk of death immediately after birth.

Key words: dog, amniotic fluid, uric acid, glucose, lactate, creatinine

1 INTRODUCTION

In mammals, fetal growth, development and well-being are regulated by several factors and the placenta and fetal fluids contribute to maintain the more suitable environment for the fetus. Amniotic fluid is a very complex and unique fluid that nourish and protect the fetus, whose composition changes widely along the pregnancy course, and also in relation to some maternal, fetal or placental imbalance or disease, and could reflect the metabolisms of all the three compartments. In humans, amniotic fluid production is mainly provided by excretions of fetal urine and the secretion from the respiratory tract and oral cavity (Brace et al.,1994; Underwood et al., 2005), although also the gastrointestinal tract and fetal skin before keratinization contribute to amniotic fluid composition (Brace et al.,1994).

In human-beings the analysis of amniotic fluid composition received, in the last fifty years, a strong scientific interest, so that many aspects were fully investigated, and markers for the correct management of both the pregnant woman and fetuses/newborns were detected. In particular, some amniotic parameters near term of pregnancy were proved to be useful for the early recognition of fetal well-being disturbances and neonatal outcome in humans (Wiberg-Itzel et al., 2014), but also in other animal species (Koski and Ferguson, 1992).

In humans, perinatal asphyxia is a common disorder, considered the most important cause of stillbirth and neonatal dead (Banupriya et al., 2008). Hypoxia induces an anaerobic metabolism, leading to the release of metabolic degradation products, such as lactic acid (Chen et al. ,2000; Liu et al., 2006), coupled to the increased production of hypoxanthine, as the result of accelerated ATP degradation and consequent increased production of purine nucleotides. When the hypoxia condition persists, hypoxanthine is oxidized to xanthine and uric acid (Bader et al., 1995; Akisü and Kültürsay, 1998). A transient, short-term asphyxia is recognized also in dogs during the process of birth, also under normal condition (Grundy, 2006). It was however recognized that during the transition from the fetal to the neonatal, extrauterine life, hypoxia is transient and recover within 60 minutes after birth (Vassalo et al., 2015). On the other hand, the process of labor in dogs could be very long, so that fetal death can reach very high percentages, as compared to other species, especially when dystocia occur (Moon et al., 2001). In addition, asphyctic puppies are more susceptible for neonatal diseases, such as septicaemia, and therefore to an increased risk for neonatal death (Johnston et al., 2001). Taken together, the birth process and the early neonatal period, represent the most challenging stages for the dogs offspring. Because of these very high percentage of perinatal losses, the full knowledge about the normal changes occurring during the last intrauterine fetal development and of the process of birth are needed to reduce the impact of canine perinatal death.

When the early neonatal outcome is concerned, beside the risk for perinatal asphyxia, also the nutrient storage and the energetic metabolism are recognized as the most important parameters

affecting neonatal survival (Vassalo et al., 2015). In humans and in other animal species, amniotic fluid is swallowed by the fetus, and some amniotic constituents, such as carbohydrates and proteins, are digested and absorbed by the fetus itself (Pitkin and Reynolds, 1975; Charlton-Char and Rudolf, 1979). Therefore, the amniotic fluid was considered as a possible source of nutrition for the fetal development and growth (Mulvihill et al., 1985). Glucose, recognized as the major metabolic fuel for the fetus (Battaglia and Meschia, 1978) and for the dog immediately after birth (Johnston et al., 2001), was showed as one of the amniotic fluid components in both humans and other animal species. In humans, the condition of intrauterine fetal growth retardation was associated to low glucose amniotic concentrations (Drazancić and Kuvacić, 1974). In addition, Koski and Fergusson (1992), reported a positive correlation between amniotic glucose concentrations and fetal weight in rats.

Beside glucose, other amniotic compounds, such as lactate and uric acid, were considered as putative indicators of fetal maturity and possible metabolic distress (Cherry et al., 1969). Koski and Fergusson (1992) demonstrated the role of some amniotic fluid constituents as predictors of the fetal metabolic status in rats. Those authors found a negative correlation between amniotic uric acid concentrations and fetal weight in rats, while amniotic lactate was not significantly associated to fetal weight. Therefore, it was stated that the measurements of amniotic uric acid could be predictable for fetal growth and metabolic maturity.

Although amniotic fluid lactate concentrations can provide information about fetal acidemia, some authors (Torrance et al., 2013), reported that a normalization on amniotic fluid volume by calculating the lactate to creatinine ratio is necessary for a more accurate estimation of fetal lacticaemia.

Creatinine, in humans, is considered one of the protein constituents of amniotic fluid, especially toward term of pregnancy, when it results from the excretional activity of the fetus.

The knowledge about the full composition of canine fetal fluids is desirable from a scientific point of view, and specifically, the detection of possible markers for a better evaluation of the neonate at

birth could be very useful from a clinical perspective. In this respect, some parameters proved to be useful for the early prediction of neonatal outcome or of the metabolic maturity or status in humans, would be interesting also for the assessment of newborn dogs.

To the authors knowledge, the normal composition of canine amniotic received little attention, and because some amniotic parameters could provide markers for the early prediction of neonatal outcome and/or of the metabolic status for a better neonatal management also in dogs, the present study was aimed to evaluate the amniotic concentration of some constituents, such as uric acid (UA), glucose (G), lactate (L), creatinine (CREA) and the lactate to creatinine ratio (L/CREA) in small-sized purebred bitches submitted to elective Caesarean section at term. The possible relation between the studied parameters and newborn outcome, as well as the possible effects played by maternal parity, newborn gender and Apgar score on UA, G, L, CREA and L/CREA were also evaluated.

2 MATERIAL AND METHODS

2.1 Animals

The study enrolled 7 primiparous (PP) and 20 multiparous (MP) small-sized (bodyweight ≤10 kg) purebred bitches, belonging to several breeds (10 Chihuahua, 5 Maltese, 3 Jack Russel terrier, 2 toy Poodle, 2 Shih-tzu, 2 Duchshound, 2 Pug and 1 miniature Bull terrier), and submitted to elective Caesarean section, at term.

Elective Caesarean section was always performed for the health of both mothers and puppies, because all the bitches belonged to breeds at high risk of dystocia or showed a previous history of troubles at parturition. All female dogs were healthy, regularly vaccinated and submitted to parasite prophylaxis, and fully monitored from the time of mating (or artificial insemination), along the complete gestation length, until parturition, as reported by Meloni et al. (2014). Pregnancy diagnosis was performed by ultrasounds at 20-28 days after the sole mating, when the estimation of parturition date was also performed by measuring the inner chorionic cavity (Beccaglia and Luvoni,

2006), and the fetal viability assessed by the detection of early fetal cardiac motion, (Davidson and Baker, 2009). A further prediction date of parturition was additionally calculated through the ultrasonographic measurement of biparietal diameter at 40-45 days of pregnancy (Beccaglia and Luvoni, 2006), and fetal viability re-evaluated. At the ultrasonographycally estimated parturition day, the elective Caesarean section was performed on those bitches with plasma progesterone (P4) concentrations ≤2 ng/ml. For bitches with plasma P4 still above 2 ng/ml, fetal viability and plasma P4 concentrations were daily checked until surgery (Meloni et al., 2014).

The study was performed, when the national regulation did not require an institutional approval for the use of waste biological materials obtained during routine clinical management of patients. Before Caesarean section, all the owners signed an informed consent, not only for the surgery, but also specifically to allow the collection and use of amniotic fluids for research purposes. The anesthesia was mainly aimed to minimize the negative impact on puppy newborns and performed following the same protocol in all the bitches, as reported by Meloni et al. (2014).

2.2 Amniotic fluids collection

The amniotic fluid samples were collected, after fetal membranes opening, from each fetus, aseptically by the amniotic sac with sterile syringe, in which the needle was withdrawal to avoid any possible touch or damage to the fetus (Meloni et al., 2014). Newborn resuscitation and amniotic fluids collection were performed by two separate operators and fluid collection never affected the prompt neonatal care and resuscitation, performed by veterinarians particularly skilled in neonatal management (Meloni et al., 2014). The collected amniotic fluids were centrifuged at 1000 x g for 20 minutes and immediately stored at -20° C until analysis, performed always within 2 months from samples collection, for UA, G, L, and CREA concentrations measurement.

2.3 Newborn evaluation

Within 5 minutes of birth, the neonates were evaluated for viability by APGAR score (Veronesi et al., 2009), gender, presence/absence of gross physical malformations, and weighed before nursing.

Body weight at birth was compared to the breed reference range reported by the Italian Kennel Club (ENCI). Newborn survival was re-checked at 24 hours after birth and at 7 days of age.

2.4 Uric acid, glucose, lactate, and creatinine analysis

After thawing, each fluid sample was re-centrifuged at 1400 x g for 10 minutes, and UA, L and CREA analyzed on the supernatant by spectrorophotometry, using an automatic analyzer Cobas Mira Classic (Roche®, Basel, Switzerland). Uric acid was analyzed by modified enzymatic colorimetric trinder (Uric Acid - Ben Biochimical Enterprise Srl, Milan, Italy, intra- and inter-assay CV 2.65 and 4.86, respectively; MDC: 0.28 mg/dl), lactate was assayed by colorimetric trinder (L-Lactate - Ben Biochimical Enterprise Srl, Milan, Italy, intra- and inter-assay CV 1.07 and 1.53, respectively; MDC: 1.50 mg/dl), and creatinine by modified Jaffè colorimetric, cinetic assay (Creatinina - Hagen Diagnostica Srl, S. Giovanni Valdarno, Italy, intra- and inter-assay CV 2.70 and 5.32, respectively; MDC: 0.09 mg/dl). Glucose was analyzed by (GOD-POD) enzymatic colorimetric method on the basis of Trinder reaction (Hagen Diagnostica srl, S. Giovanni Valdarno, Italy, intra- and inter-assay CV 2.01 and 3.82, respectively; MDC: 6.97 mg/dl).

2.5 Statistical analysis

Data about UA, G, L, and CREA amniotic concentrations, as well as L/CREA ratio, in relation to newborn survival and the possible effect of maternal parity, newborn gender, and statistically analyzed by a non parametric ANOVA test (Wilcoxon test), while the possible effect of the Apgar score was assessed using the Kruskall-Wallis test using JMP7 (SAS Inc, Cary, NC). Significance was set for P value <0.05.

3 RESULTS

3.1Clinical findings

From the 27 bitches submitted to elective Caesarean section, a total of 74 puppies were born, 58 from multiparous and 16 from primiparous bitches, 38 females and 36 males; litter size ranged between 1 and 6, with an average of 3.4 puppies per litter. Out of 74 puppies, 68 (92%) of them were normally developed, with Apgar score ≥ 7, without gross birth defect, normal weighed and

survived at 24 hours and at 7 days of age, while 6 (8%) puppies belonging to different bitches were severely depressed (Apgar < 4) and did not survive at 24 hours after birth. For the 6 dead puppies abnormal maternal behaviors or illness were excluded, and necropsy did not evidence gross malformations, trauma or infections.

3.2 Amniotic fluid UA, G, L, CREA, and L/CREA

A total of 68 amniotic samples were collected from normal, surviving newborns, and 6 amniotic samples were collected from the less viable puppies dying within 24 hours after birth. All the 74 amniotic samples were analyzed for UA, G, L, and CREA concentrations and L/CREA calculated.

Although the number of puppies not surviving at 24 hours after birth was little (N=6), a statistical comparison between amniotic UA, G, L, and CREA concentrations and L/CREA (median and 25-75 percentiles) in the 68 normal, surviving puppies and in the 6 less viable puppies dead within 24 hours after birth, was performed (Table 1).

Data about the median and 25-75 percentiles amniotic UA, G, L, CREA, and L/CREA measured on the 68 amniotic samples belonging to normal, surviving puppies retrieved from the 27 litters, and grouped in relation to maternal parity, as well as according to newborn gender, are reported in Table 2.

In Table 3 data about the median and 25-75 percentiles amniotic UA, G, L, CREA, and L/CREA measured on the 68 amniotic samples belonging to normal puppies, were grouped according to the Apgar score.

4 DISCUSSION

To the authors knowledge, in comparison to humans, the fetal fluids composition in dogs remains still not completely investigated. The full knowledge of the normal composition, and the detection of some possible markers for the early prediction of neonatal outcome and/or of the metabolic status for a better neonatal management also in dogs, are thus desirable.

A previous study reported the amniotic glucose, lactate and cortisol concentrations in puppies born by bitches belonging to several, different sized, bitches and by different type of parturition

(Groppetti et al, 2015). However, data on a more homogeneous group and also about some other parameters such as, UA, Crea and the L/CREA were not reported.

Therefore, the present study was designed to depict the concentrations of UA, G, L, and CREA, and the L/CREA in amniotic fluid samples collected from newborn puppies, born by elective Caesarean section from healthy, small-sized bitches at term of a normal course pregnancy, in relation to newborn survival and assessing the possible effect played by maternal parity, neonatal gender and Apgar score on amniotic UA, G, L, and CREA concentrations, and on the L/CREA.

Although the number of puppies dead within 24 hours after birth was little (N=6) the statistical comparison between data obtained on amniotic fluids collected from those puppies and surviving subjects was performed because, according to the implied purpose of the present study, from a clinical perspective, it could be useful to detect possible markers for the early prediction of newborn outcome or metabolic imbalance. However, the authors highlights that these data must be considered only preliminary and deserve further investigations on a suitable number of puppies. From this comparison, the most interesting result is the lower (about one-half) median amniotic glucose concentrations ($p<0.05$) in non surviving puppies respect newborns surviving at 24 hours, and at 7 days of age. However, because 2 of this 6 amniotic glucose concentrations were below the minimum detectable concentration, this result must be considered very cautiously, and further studies are needed to define the real amniotic glucose concentrations in puppies at risk for death immediately after birth.

However, it seems reasonable to suppose that amniotic glucose concentrations in non surviving puppy could be much lower than that found for surviving puppies. Because the 6 dead puppies showed a normal birth weight, in relation to the belonging breed, it seems possible to hypothesize that the low amniotic glucose concentrations could be the cause, or the effect, of metabolic imbalance at the time of birth, and could be suspected as at least one of the causes of death. However, even if no significant differences were found for the other studied parameters, based on these very preliminary results, it is not possible to exclude the involvement of other factors,

responsible for the neonatal adaptation impairment. In a previous study (Groppetti et al., 2015), stillborn mean amniotic concentrations were found lower (14.2±0 mg/dl) in comparison to puppies alive at 48 hours after birth (20.4±9.1 mg/dl).

The results about median amniotic glucose concentrations in surviving puppies (18 mg/dl) was similar to the mean value (20.4 mg/dl) reported by Groppetti et al. (2015), while in dead puppies the median glucose concentrations in the present study were lower as compared to the mean value reported by those authors (7 vs 14.2 mg/dl).

Although gathered from a small number of puppies, the lack of significant differences in uric acid, lactate, creatinine and lactate to creatinine ratio between surviving and not surviving puppies is interesting and seems to suggest that asphyxia and renal maturity could be less substantial than glucose as metabolic fuel, for the early puppies survival.

When data obtained on the 68 amniotic samples collected from the normal, surviving puppies are considered in relation to the Apgar score, no significant association was found between the studied parameters and the Apgar score, in agreement with results reported by Groppetti et al (2015).

Within the group of surviving puppies, the overall UA median concentration (0.97 mg/dl) was lower than the concentrations reported in amniotic samples collected in human normal pregnancies at term (5.8±1.2%) (Cherry et al., 1969), and difficult to compare to data showed by Fresno et al. (2012) in cats at term, that refer to values <5 mg/dl. None effect of maternal parity, newborn gender and Apgar score was observed on UA amniotic concentrations.

The overall amniotic median glucose concentration (18 mg/dl) was very similar to data reported for amniotic mean glucose in human normal pregnancies (22±12 mg/dl) (Mohajeri et al., 2009), but lower than glucose mean amniotic concentrations (69 mg/dl) reported for cats at 60 days of pregnancy (Fresno et al., 2012). Although no differences were found in dependence of maternal parity and Apgar score, amniotic median glucose concentrations were surprisingly significantly higher ($p<0.05$) in male than in female puppies (25 vs 15 mg/dl, respectively), difference not reported for humans and not investigated in cats by Fresno et al. (2012), and by Groppetti et al.

(2015) in newborn dogs. Koski and Fergusson (1992), reported that the amniotic glucose concentration might predict metabolic maturity, so that because in the present study all the newborns were healthy and viable, and survived at 24 hours and at 7 days of age, it seems possible to suggest that female puppies could be considered at a "lower" metabolic maturity in comparison to males.

The overall amniotic median lactate concentration (72 mg/dl) was a bit higher than data reported in humans during normal labour (7.1±1.45 mmol/L, corresponding to 64±13.06 mg/dl) (Korenovsky et al., 2013), but lower in comparison to data (12.5 mmol/l, corresponding to 112.6 mg/dl) reported for late pregnant cats by Fresno et al. (2012). The amniotic lactate concentrations was also studied in horses (Pirrone et al., 2012), and the mean concentrations reported for foals born from spontaneous eutocic parturition were higher (14.47 mmol/L, corresponding to 130.36 mg/dl) in comparison to data observed in the present study in dogs born by elective Caesarean section. The lower amniotic lactate concentrations observed in the present study in the dogs in comparison to data reported for horses were, however, not unexpected. In fact, in the present study, dogs were submitted at elective Caesarean section before the start of labor, while data reported for horses were obtained in amniotic samples collected during spontaneous parturition, when foaling was already started. However, it is interesting to note that the amniotic lactate concentrations in dogs resulted a bit higher than data reported for women during normal labor, suggesting, beside a specie-specific effect, also an effect played by the type of parturition. Lactate mean amniotic concentration in puppies born by elective Caesarean section reported by Groppetti et al. (2015) (8.7±3.4 mmol/l, corresponding to 78±30.6 mg/dl) were very similar to the median values found in the present study. In horses a negative correlation between amniotic lactate concentrations and parity was found. In the present study, no effects played by maternal parity, newborn gender and Apgar score on amniotic lactate concentrations were found.

The overall median creatinine concentrations was 1.7 mg/dl, very similar to data reported in humans under normal conditions (1.9±1.5 mg/dl) (Cherry et al., 1969), but lower than creatinine

concentrations showed by Fresno et al. (2012) for cats at 60 days of pregnancy (<10 mg/dl). Creatinine output is considered as a good indicator of renal function and, namely, of glomerular filtration rate, and its reduced concentration in human amniotic fluid associated to maternal diabetes mellitus and toxemia. In the present study, the CREA amniotic concentrations could be supposed to be related to the presence of fetal urines also in the amniotic compartment, due to the final intrauterine development of the urinary system and the deviation of fetal urines from the allantoic sac toward the amniotic compartment through the urethra as the urachus progressively occludes (Fresno et al., 2012). However, the percentile values seems to suggest that, upon the results from the present study, a rather wide range of CREA amniotic levels should be considered as indicative of normal maternal and fetal conditions in dogs. Unfortunately the statistical analysis failed to detect differences in creatinine amniotic concentrations according to maternal parity, and in relation to newborn gender and Apgar score, so that other parameters should be investigated in order to define what could influence such a wide range of normal CREA amniotic concentrations in dogs.

According to Torrance et al. (2013) for a better evaluation of fetal lactacemia, the amniotic lactate concentration was normalized by the calculation of the L/CREA ratio. In the present study the overall L/CREA ratio was 41 and none significant difference was found according to newborn gender or Apgar score. As expected, also this ratio showed a marked wide percentiles and, as a consequence, a wide range of normality. Higher L/CREA median ratio in amniotic fluid collected from puppies born by multiparous in comparison to primiparous bitches (46 vs 25, respectively) was found, but the biological meaning of this finding deserves further investigations.

In conclusion, data from the present study showed that, in purebred small-sized dogs, amniotic glucose concentrations could represent a suitable parameter for the detection of puppies at risk for death within 24 hours after birth, while lactate, creatinine and lactate to creatinine do not seem to assume a relevant role. Within normal, surviving puppies, lower amniotic glucose concentrations are found in female as compared to male puppies, while a higher lactate to creatinine ratio was found in amniotic fluids of puppies born by multiparous respect to primiparous bitches. However,

the wide ranges observed for each parameter, suggesting a wide inter-individual variation, deserve further investigations to define factors affecting the wide range of normality.

Table 1- Amniotic uric acid, glucose, lactate, and creatinine concentrations and lactate/creatinine (median and 25-75 percentiles), in the 68 samples collected from the normal, surviving puppies, and in the 6 amniotic samples collected form puppies dead within 24 hours after birth

	Amniotic fluid				
	Uric acid (mg/dl)	Glucose (mg/dl)	Lactate (mg/dl)	Creatinine (mg/dl)	Lactate/Creatinine
Normal (n=68)	0.97 (0.63-1.56)	18 (12-30)[a]	72 (53.3-91.2)	1.7 (1.1-3.1)	41 (22.2-63.1)
Dead (n=6)	1.18 (1-1.67)	7 (0.5-11.7)[b]	52 (51.5-59.4)	2.1 (1.6-3.8)	26 (13.4-39.2)

[a,b]Different superscript within column refers significant differences ($p<0.05$)

Table 2 - Amniotic uric acid, glucose, lactate, and creatinine concentrations and lactate/creatinine (median and 25-75 percentiles), in the 68 amniotic samples belonging to normal, surviving puppies, grouped in relation to maternal parity and to newborn gender

	Amniotic fluid				
	Uric acid (mg/dl)	Glucose (mg/dl)	Lactate (mg/dl)	Creatinine (mg/dl)	Lactate/Creatinine
PP (n=15)	1.2 (1.02-1.96)	15 (3.5-19)	63 (51-85.2)	2.3 (1.6-3.5)	25 (19-35.5)[a]
MP (n=53)	0.9 (0.57-1.48)	19 (14-30.7)	73 (59.6-91.2)	1.6 (1.1-2.5)	46 (23.2-66.5)[b]
Males (n=33)	0.91 (0.65-1.3)	25 (17-31)[a]	66 (51.8-84.2)	1.7 (1.1-3.5)	32 (20.3-57.6)
Females (n=35)	1.03 (0.63-1.98)	15 (8-26)[b]	76 (63-93)	1.7 (1.1-2.6)	48 (26.3-71.7)

[a,b]Different superscript within column refers significant differences ($p<0.05$)

Table 3 – Amniotic uric acid, glucose, lactate, and creatinine concentrations and lactate/creatinine (median and 25-75 percentiles), in the 68 amniotic samples belonging to normal, surviving puppies, grouped in relation to Apgar score

	Uric acid (mg/dl)	Glucose (mg/dl)	Lactate (mg/dl)	Creatinine (mg/dl)	Lactate/Creatinine
Apgar 7 (N=14)	0.91 (0.63-1.77)	15 (10.3-18)	62 (43.5-74.3)	1.7 (1.1-3.3)	39 (15.2-56.5)
Apgar 8 (N=29)	1.08 (0.83-1.69)	19 (9-35)	77 (63.7-97.3)	2.0 (1.2-2.8)	36 (23.3-64.4)
Apgar 9 (N=20)	0.89 (0.64-1.55)	16 (14-26.5)	76 (63.8-86.5)	1.8 (1.2-3)	36 (20.3-68.7)
Apgar 10 (N=5)	0.97 (0.86-1.12)	25 (12-26)	77 (44.4-84)	1.7 (1.6-7.9)	46 (4.6-51.9)

REFERENCES

Akisü, M., Kültürsay, N. (1998). Value of the urinary uric acid to creatinine ratio in term infants with perinatal asphyxia. **Acta Paediatr Jpn** 40, 78-81

Bader, D., Gozal, D., Weinger-Abend, M., Berger, A., Lanir, A. (1995). Neonatal urinary uric acid/creatinine [correction of creatinine] ratio as an additional marker of perinatal asphyxia. **Eur J Pediatr** 154, 747-749

Banupriya, C., Ratnakar, Dourerdjou, P., Mondal, N., Bhat Vishnu, Koner, BC. (2008). Can urinary excretion rate of malondialdehyde, uric acid and protein predict the severity and impending death in perinatal asphyxia? **Clin Biochem** 41, 968-973

Battaglia, F.C., Meschia, G. (1978). Principal substrates of fetal metabolism. **Physiol Rev** 58, 499-527

Beccaglia, M., Luvoni, G.C. (2006). Comparison of the accuracy of two ultrasonographic measurements in predicting the parturition date in the bitch. J Small Anim Pract 47, 670–673

Brace, R.A., Wlodek, M.E., Cock, M.L., Harding, R. (1994). Swallowing of lung liquid and amniotic fluid by the ovine fetus under normoxic and hypoxic conditions. Am J Obstet Gynecol 171, 764-770

Charlton-Char, V., Rudolf, A. (1979). Digestion and absorption of carbohydrates by the fetal lamb in utero. Pediatr Res 13, 1018-1023

Chen, H.J., Yau, K.I.T., Tsai, K.S. (2000). Urinary uric acid/creatinine ratio as an additional marker of perinatal asphyxia. JFMA 99, 771-774

Cherry, S.H., Dolger, H., Rosenfield, R.E., Kochwa, S. (1969). Amniotic fluid urea nitrogen, uric acid, and creatinine in diabetic pregnancies. B New York Acad Med 45, 46-52

Davidson, A.P., Baker, T.W. (2009). Reproductive ultrasound of the bitch and queen. Top Companion Anim M 24, 55-63

Drazancić, A., Kuvacić, I. (1974). Amniotic fluid glucose concentration. Am J Obstet Gynecol 120, 40-48

Fresno, L., Rodriguez-Gil, J.E., Rigau, T., Pastor, J., Rivera del Alamo, M.M. (2012). Modulation of the biochemical composition of amniotic and allantoic fluids as a control mechanism of feline foetal development. Placenta 33, 522-527

Groppetti, D., Martino, P.A., Ravasio, G., Bronzo, V., Pecile, A. (2015). Prognostic potential of amniotic fluid analysis at birth on canine neonatal outcomes. Vet J 206, 423-425

Grundy, S.A. (2006). Clinically relevant physiology of the neonate. Vet Clin N Am-Small 36, 443-459

Johnston, S.D., Kustritz, V.R., Olson, P.N.S. (2001). The neonate-from birth to weaning. In: Canine and Feline Theriogenology. Saunders, Philadelphia. pp 146-167

Korenovsky, Yu.V., Chugunova, T.N., Filchakova, O.N., Sinelnikova, L.M., Shabalina, Yu.V., Elchaninova, S.A. (2013). Lactate Concentration in Amniotic Fluid at Early Neonatal Period in Perinatal Hypoxia. Kazanskiy Meditsinskiy Zhurnal 5, 704-706

Koski, K.G., Fergusson, M.A. (1992). Amniotic fluid composition responds to changes in maternal dietary carbohydrate and is related to metabolic status in term fetal rats. **J Nutr** 122, 385-392

Liu, L., Zhou, H.Y., Song, J.W., Nie, W.X., Su, Z.Y. (2006). Measurement of urinary S100B protein levels and lactate/creatinine ratio in early detection of neonatal hypoxic-ischemic encephalopathy. **WPJ** 2, 270-275

Meloni, T., Comin, A., Rota, A., Peric, T., Contri, A., Veronesi, M.C. (2014). IGF-I and NEFA concentrations in fetal fluids of term pregnancy dogs. Theriogenology 81, 1307-1311

Mohajeri Tehrani, M., Amini Moghaddam, S., Annabestani, Z., Heshmat, R., Alyasin, A., Aghaie Meibodi, H., Larijani, B. (2009). Amniotic fluid, maternal, and neonatal serum C-peptide as predictors of macrosomia: A pilot study. J Diabetes Metab Disord 8, 129-136

Moon, P.F., Massat, B.J., Pascoe, P.J. (2001). Neonatal critical care. Vet Clin N Am-Small 31, 343-366

Mulvihill, S.J., Stone, M.M., Debas, H.T., Fonkalsrud, E.W. (1985). The role of amniotic fluid in fetal nutrition. J Pediatr Surg 20, 668-672

Pirrone, A., Mariella, J., Gentilini, F., Castagnetti, C. (2012). Amniotic fluid and blood concentrations in mares and foals in the early postpartum period. Theriogenology 78, 1182-1189

Pitkin, R.M., Reynolds, W.A. (1975). Fetal ingestion and metabolism of amniotic fluid protein. Am J Obstet Gynecol 123, 356-363

Torrance, H.L., Pistorius, L., Voorbij, H.A., Visser, G.H. (2013). Lactate to creatinine ratio in amniotic fluid: a pilot study. **Journal of Maternal-Fetal and Neonatal Medicine** 26, 728-730.

Underwood, M.A., Gilbert, W.M., Sherman, M.P. (2005). Amniotic fluid: not just fetal urine anymore. **J Perinatol** 25, 341-348

Vassallo, F.G., Simões, C.R., Sudano, M.J., Prestes, N.C., Lopes, M.D., Chiacchio, S.B., Lourenco, M.L. (2015). Topics in the routine assessment of newborn puppy viability. Top Companion Anim M 30, 16-21

Veronesi, M.C., Panzani, S., Faustini, M., Rota, A. (2009). An Apgar scoring system for routine assessment of newborn puppy viability and short-term survival prognosis. Theriogenology 72, 401-407

Wiberg-Itzel, E., Pembe, A.B., Wray, S., Wihlbäck, A.C., Darj, E., Hoesli, I., Åkerud, H. (2014). Level of lactate in amniotic fluid and its relation to the use of oxytocin and adverse neonatal outcome. Acta Obstet Gynecol Scand 93, 80-85

CHAPTER 10

Biochemical composition of fetal fluids in at term, normal developed, healthy, viable dogs and preliminary data from pathologic littermates

BIOCHEMICAL COMPOSITION OF FETAL FLUIDS IN AT TERM, NORMAL DEVELOPED, HEALTHY, VIABLE DOGS AND PRELIMINARY DATA FROM PATHOLOGIC LITTERMATES

In the last years, the interest for the study of canine neonatology aimed to improve knowledge regarding perinatal physiology in puppies and to reduce perinatal mortality, is increasing. In fact, perinatal mortality in dogs can reach peacks up to 30% with a relevant economic loss for breeders (Indrebø et al, 2007; Tønnessen et al, 2012); this mortality rate can be reduced by a proper assistance during the process of birth and also during the neonatal period.

One of the most important events in early gestation is the formation of extra-embryonic membranes, essentials for the feto-maternal exchanges. Fetal fluids provides essential nutrients and molecules for the growing fetus. Amniotic fluid originates from the respiratory and gastrointestinal tract and from the fetal skin before keratinization (Fresno et al, 2012). Allantoic fluid is composed primarily by excretion of fetal kidneys.

In humans, the amniotic fluid, contained in the amnion cavity, is essential for fetal development and maturation during pregnancy (Xing-long Tong et al, 2009). The composition of amniotic fluid in humans was widely investigated and many studies were done in order to detect biomarkers of pregnancy-associated abnormalities (Xing-long Tong et al, 2009) (Gulbis et al, 1998) (Daniel et al, 2004). In particular, different studies were performed on the biochemical composition of amniotic fluid in humans during pregnancy and at term, also by the comparison of amniotic fluid composition with maternal serum and umbilical cord serum. Those studies evidenced that the electrolyte content is similar between amniotic fluid and maternal and umbilical cord serum, while the concentration of organic (total protein, glucose, triglyceride, cholesterol and enzymes) substances was lower in amniotic fluid as compared to maternal and umbilical cord serum. (Bala et al, 1986; Underwood et al, 2005; Ch et al, 2007; Xing-long Tong et al, 2009), suggesting that the

composition of amniotic fluid is not the result of the simple filtration from the blood but an independent fluid.

Amniotic and allantoic fluid biochemical composition was also investigated in domestic animals, in particular in sheeps, goats, horses, bovines and also in cats.

Studies in mares evidenced some differences between amniotic and allantoic fluid composition, and point out changes in fetal fluids composition during pregnancy. A study from Kochhar et al (1997) investigated differences in fetal fluids biochemical composition in relation to the type of parturition, in this study a significantly higher alkaline phosphatase (AKP), lactate dehydrogenase (LDH); Bilirubin, and urea (BUN) and lower acid phosphatase (ACP) were observed in allantoic fluid of mares with dystocia as compared with normal foaling ones; the concentration of bilirubin and AKP in amniotic fluid of mares with dystocia were significantly higher when compared with foals with normal foaling.

In ruminants amniotic fluid biochemical composition was investigated among pregnancy in ewes at 70, 100, 145 days of pregnancy by Prestes et al (2001) and theresults were similar to those reported by previous studies (Mellor and Salter 1972; Tomoda et al, 1987; Aidasani et al, 1992; Cock et al, 1994; Lovell et al, 1995) with a change of pH, glucose, urea, creatinine, gamma GT, sodium, potassium, chloride and protein during pregnancy. Studies in goats evidenced changes in electrolyte, enzymes and metabolites in amniotic and allantoic fluid and also in maternal serum during different stages of pregnancy (Khadjeh et al, 2007; Saleh Tabatabaei and Morteza Mamoei, 2012). Also in cattle the concentration of enzymes, electrolyte and metabolites in fetal fluids and maternal blood were analyzed during pregnancy evidencing changes in the concentrations during pregnancy (Baetz et al, 1976; Saleh Tabatabaei and Morteza Mamoei 2012). All these studies demonstrated that the changes in fetal fluids composition probably reflects changes in transport

and metabolic activity as well as different contributions of the fetal and placental tissues to the fetal fluids composition. As already reported the feto-placental unit is a dynamic system that causes constant exchanges of water and fetal fluids components between the fetal fluids compartments and the maternal blood, which are reflected in changes in chemical, biochemical and physical constituents of fetal fluids (Aidasani et al, 1993).

More recently the interest of fetal fluids composition involved also the domestic cat, in fact a study by Fresno et al (2012) investigated the fetal fluids biochemical composition in cats during pregnancy and at term. Fetal fluids were collected at days 30, 40, 50 and 60 of pregnancy from pregnant queens after ovariohysterectomy. Thats study evidenced that, also in cats, the composition of fetal fluids is not the result of simple filtration from maternal blood and the fetus itself plays an important role in the final biochemical composition of both fluids throughout pregnancy. In cats, the composition of amniotic and allantoic fluids is only slightly different between the two fluids, due to the possible diffusion from allantois vessels to amniotic cells. The concentration of electrolyte, metabolites and enzymes changes during pregnancy as the result of changes in metabolic and transport activity and differences in the contribution of the fetus and the placenta in fetal fluids composition.

To the author knowledge, despite the scientific interest on fetal fluids composition in humans and also in animals, this topic received little interest, until recently, in dogs.

In fact only few studies investigated the composition of amniotic and allantoic fluid in dogs and no studies provided the biochemical composition of fetal fluids. Although fetal fluids are easily collectable in dogs during parturition, as already reported by Meloni et al (2014) and Dall'Ara et al (2015).

Therefore, because knowledge about perinatal physiology in dogs are scarce, the investigation of fetal fluids biochemical composition could provide information on the last intrauterine fetal stages of development.

REFERENCES

Aidasani R, Chauhan RAS, Tiwari S, Shukla SP. (1993) Studies on electrolytes of caprine fetal fluids and fetal serum. Indian Vet J 70:239–241

Aidasani R, Pieterse ME, Van Derweyden GG, Taverne MAM. (1992) Some metabolic constituents of caprine foetal fluids and foetal serum. Indian J. Anim. Sci., 62 (1992), pp. 335-336

Baetz AL, Hubbert WT, Gram CK. (1976) Changes of biochemical constituents in bovine foetal fluids with gestational age. Am j vet res, 37: 1047-1052.

Bala S, Seth S, Seth PK. (1986) Total proteins in human amniotic fluid. Aust N Z J Obstet Gynaecol. 1986 May;26(2):141-4.

Cho CK, Shan SJ, Winsor EJ, Diamandis EP. (2007) Proteomics analysis of human amniotic fluid. Mol Cell Proteomics. 2007 Aug;6(8):1406-15. Epub 2007 May 10.

Cock ML, Wlodek ME, Hooper SB, McCrab GJ, Harding R. (1994) The effects of twenty-four hours of reduced uterine blood flow on fetal fluid balance in sheep. Am. J. Obstet. Gynecol., 170 (1994), pp. 1442-1451

Dall'Ara P, Meloni T, Rota A, Servida F, Filipe J, Veronesi MC. (2015) Immunoglobulins G and lysozyme concentrations in canine fetal fluids at term of pregnancy. Theriogenology 2015; 83:766-71.

Fresno L, Rodriguez-Gil JE, Rigau T, Pastor J, Riviera del Alamo MM. (2012) Modulation of the biochemical composition of amniotic and allantoic fluids as a control mechanism of feline foetal development. Placenta 2012;33:522-7.

Gulbis B, Gervy C, Jauniaux E. (1988) Amniotic fluid biochemistry in second-trimester trisomic pregnancies: relationships to fetal organ maturation and dysfunction. Early Hum Dev. 1998 Oct;52(3):211-9.

Indrebø A, Trangerud C, Moe L. (2007) Canine neonatal mortality in four large breeds. Acta Vet Scand 49(Suppl I), S2.

Khadjeh GhH, Ranjbar R, Salehi M, Banankhojasteh SM. (2007) Biochemical evaluation of amniotic fluid during different stages of gestation in the goat. Iranian J Vet Res 8:266–269

Kochhar HPS, Simran PS, Nanda AS, Kaur R. (1997) Comparative biochemical indices of fetal fluids in normal foaling and stressful delivery in Indian thoroughbred mares. Journal of Equine Veterinary Science, Volume 17, Issue 4, April 1997, Pages 206-210

Lovell KL, Sprecher DJ, Ames NK, Jones MZ. (1995) Development and efficacy of ultrasound-guided fetal fluid aspiration techniques for prenatal diagnosis of caprine β-mannosidosis. Theriogenology, 44 (1995), pp. 517-527

Mellor DJ, Slater JS. (1972) Daily changes in foetal urine and relationship with amniotic and allantoic fluid and maternal plasma during the last two months of pregnancy in conscious, unstressed ewes with chronically implanted catheters. J. Physiol., 227 (1972), pp. 503-525

Meloni T, Comin A, Rota A, Peric T, Contri A, Veronesi MC. (2014) IGF-I and NEFA concentrations in fetal fluids of term pregnancy dogs. Theriogenology 2014; 81:1307-11.

of the fetal fluids and maternal blood serum in goat. Comp Clin Pathol (2012) 21:1305–1312.

Prestes NC, Chalhoub MC, Lopes MD, Takahira RK. (2001) Amniocentesis and biochemical evaluation of amniotic fluid in ewes at 70, 100 and 145 days of pregnancy. Small Rumin Res. 2001 Mar;39(3):277-281.

Saleh Tabatabaei and Morteza Mamoei. (2012) Changes in the biochemical composition of fetal fluids and maternal blood serum during different days of gestation in cattle. Comp Clin Pathol (2012) 21:1005–1012.

Saleh Tabatabaei. (2012) Gestational variations in the biochemical composition

Tomoda S, Brace RA, Longo LD. (1987) Amniotic fluid volume regulation: basal volumes and responses to fluid infusion withdrawal in sheep. Am. J. Physiol., 252 (1987), pp. 380-387

Tong XL, Wang L, Gao TB, Qin YG, Qi YQ, Xu YP. (2009) Potential function of amniotic fluid in fetal development---novel insights by comparing the composition of human amniotic fluid with umbilical cord and maternal serum at mid and late gestation. J Chin Med Assoc. 2009 Jul;72(7):368-73. doi: 10.1016/S1726-4901(09)70389-2.

Tønnessen R, Borge KS, Nødtvedt A, Indrebø A. (2012) Canine perinatal mortality: a cohort study of 224 breeds. Theriogenology 2012; 77:1788-801.

Underwood MA, Gilbert WM, Sherman MP. (2005) Amniotic fluid: not just fetal urine anymore. J Perinatol 25, 341-348.

CHAPTER 11

Biochemical composition of fetal fluids in at term, normal developed, healthy, viable dogs and preliminary data from pathologic littermates

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Biochemical composition of fetal fluids in at term, normal developed, healthy, viable dogs and preliminary data from pathologic littermates

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ABSTRACT

A proper canine neonatal assistance, required to reduce the high perinatal loss rate, imply a full knowledge about the fetal-to-neonatal physiology. Because fetal fluids play an important role throughout mammals pregnancy, influencing fetal growth and development, fetal well being, and contributing to guarantee the most suitable environment for the fetus, the knowledge about fetal fluids biochemical composition is of major importance. At first, the biochemical composition of fetal fluids collected by normal developed, healthy and viable newborns, is necessary to depict the normal features, and represent the first step for the further detection of abnormalities associated to fetal/neonatal distress and useful for the early identification of newborns needing special attention, immediately after birth. The present study was aimed to define the biochemical composition of amniotic and allantoic fluids collected from fetus delivered by caesarean section at term of pregnancy. To reduce the possible confounding effect of maternal labor or troubles at parturition, fetal fluids were collected only from puppies born by elective caesaeran section, at term of normal pregnancies. Fetal fluids from 76 puppies, 70 normal and six pathologic newborns, born by elective caesarean section were collected and analyzed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, lactate dehydrogenase (LDH), creatine-kinase (CK), alkaline phosphatase (ALP), creatinine, urea, amylase, lipase, gamma-glutamyl transferase (γ-GT), triglycerides, cholesterol, total proteins, albumin, globulins, glucose, magnesium, potassium, chloride, sodium, calcium, phosphorus and osmolarity. No significant differences were found between biochemical composition of amniotic or allantoic fluid in normal and pathologic newborns, maybe due to the small number of the pathologic puppies. Although some correlations between the two fluids were found (albumin, phosphorus, glucose and triglycerides), the results showed significant differences between the amniotic and allantoic biochemical composition (for all the parameters, except of alanine aminotransferase, triglycerides, cholesterol, albumin, amylase and glucose), suggesting that diverse sources could concur to the final composition of each fluid. A wide variability within and among litters was found for both amniotic and allantoic biochemical composition, and for some parameters an influence of breed body size (amniotic amylase, cholesterol, and allantoic calcium and glucose), maternal parity (amniotic and allantoic CK, glucose, LDH, chloride) and newborn gender (allantoic phosphorus) was found. Further investigations are needed for addressing the origin of each fetal fluid biochemical composition in the dog and also to indeep possible differences in fetal fluids biochemical composition between normal and pathologic puppies, providing potential markers for the quick identification of newborns that need special surveillance and cares immediately after birth.

1. Introduction

In the last years canine perinatology received an increasing interest, mainly aimed to a better management of newborn puppies and to reduce the perinatal loss rate, currently too high. In the dog species, that give birth to multiple neonates, the process of

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parturition can lasts several hours, affecting the newborns survival rate [1]. This is in contrast to the need of the as high as possible puppies survival rate, due to the economic value of breeding dogs, but also to the emotional impact of the birth process in pet dogs. In this species, the perinatal mortality refers to the sum of fresh born dead puppies plus puppies dying during the early neonatal period, considered as the first week of life [2]. The canine perinatal mortality rate is reported to be very variable in the different studies, ranging between 8% and 26% [2–4], with a considerable impact for the breeders.

Canine perinatal mortality could be limited by a correct management of whelping coupled to a proper neonatal assistance [5]. The proper canine neonatal assistance requires, in turn, the full knowledge about fetal-to-neonatal physiology to allow, on one hand, the correct management of the normal newborns, but also for the soon recognition of those puppies needing special surveillance or cares after birth. Fetal fluids, in example, recognized to play an important role throughout mammals pregnancy, influencing fetal growth and development, fetal well being, and contributing to guarantee the most suitable environment for the fetus, are scantly studied in dogs.

In humans, the origin and composition of amniotic fluid are fully known, and markers related to some maternal, fetal or placental diseases were identified, with relevant clinical impact.

Similarly to humans and sheeps, also in dogs, it may be assumed that the amniotic fluid is composed by several secretions from the urinary system, the respiratory tract, the gastrointestinal system and the skin before keratinization [6,7], while the allantoic fluid, less investigated in humans, is supposed to be mainly provided by the fetal urines, due to the final urethral canalization in the fetus.

To the authors knowledge, despite the wide scientific literature about human amniotic fluid composition, only few studies focused on the canine fetal fluid composition, although, fetal fluids can be collectible at the time of caesarean section, as previously reported by Ref. [8], without interfering with neonatal assistance. The availability of normal data about the biochemical fetal fluids composition will not only add some lacking information about dogs perinatology, but will also represent the first step for a further detection of differences between normal and pathologic puppies, providing potential markers for the quick identification of newborns at risk, that need special surveillance and cares, immediately after birth.

For all the above mentioned reasons, the aim of the present study was to report the biochemical composition of fetal fluids collected from at term, normal developed, healthy, viable puppies and the preliminary data from pathologic littermates. To reduce the possible confounding effect of maternal labor or troubles at parturition, fetal fluids were collected only from fetuses delivered by elective caesarean section, at term of normal pregnancies. The possible effect played by some maternal and fetal parameters on the normal biochemical fetal fluids composition was also assessed.

2. Materials and methods

2.1. Clinical data

The study was performed on 24 purebred bitches, belonging to several breeds. According to breed body weight, the bitches were classified, according to Federation Cynologique Internationale, as belonging to small sized breeds (body weight < 10 kg, n = 13), or large breeds (body weight > 20 kg, n = 11). No bitches belonging to medium sized (body weight 10-20 kg) breeds were enrolled.

All the bitches, were regularly vaccinated and dewormed, and healthy from the time of mating, throughout pregnancy, until parturition.

All the enrolled bitches belonged to breeds at high risk of dystocia or had previous history of troubles at whelping. Therefore, in all cases, an elective caesarean section (CS) at term of pregnancy was planned, to ensure the health of mothers and puppies.

The date of elective CS was estimated based upon the measurement of progesterone plasma concentration at mating, coupled to the prediction of parturition date by the ultrasonographic measurement of the inner chorionic cavity and biparietal diameter of the fetal skull, as reported by Meloni et al. [8]. However, CS was performed only on the base of progesterone plasma concentrations ≤2 ng/ml [8].

The same anesthesia and surgical protocol was performed in all bitches, and mainly aimed to minimize the negative impact on puppy newborns, as reported by Meloni et al. [8]. Briefly, after premedication with metoclopramide, cefazolin and oxygen mask, induction was obtained by propofol infusion and lidocaine used for infiltration on the site of surgical incision, followed by anesthesia maintenance using isofluorane in oxygen. The caesarean section was performed by ventral midline laparotomy and, only after all the fetuses were removed, tramadol and oxytocin were injected to the dam [8].

Newborn viability was assessed within 5 min after birth as reported by Veronesi et al. [1], and three levels of newborn distress identified: no distress for Apgar score between 10 and 7, moderate distress for score between 6 and 4, and severe distress for score between 3 and 0.

Each puppy was additionally evaluated for gender, absence of gross physical defects and weighed before nursing. Puppies born alive and classified as viable (absence of neonatal distress as evidenced by an Apgar score ≥7), normal weighed and without malformations were considered as “normal”, while puppies born dead, less viable, underweighted or malformed were considered as “pathologic”. Birth body weight was compared to the reference range reported for each breed by the Italian Kennel Club (ENCI).

Newborns survival was checked at 24 h of age and at 7 days after birth.

2.2. Fetal fluids collection

The study was performed during the year 2015, when the national regulation did not require an institutional approval for the use of waste biological materials, obtained during routine clinical management of patients, such as bitches requiring elective CS.

Before surgery the owners signed an informed consent, to specifically allow the collection and use of fetal fluids for research purposes.

In order to avoid every possible interference of fetal fluids collection on the neonatal first cares and reanimation procedure, fetal fluids collection and puppies cares and reanimation were always performed by two different veterinarians.

For each puppy, the amniotic and the allantoic fluids were separately collected as previously reported [8], and without any disturbance for the newborns. In some cases, the allantoic fluid was lost during the fetal bags opening procedures. Immediately after collection, fetal fluids were centrifuged at room temperature at 1000 × g for 10 min, and the separated supernatant frozen at −20 °C until analysis, performed within 3 months from fluids collection.

2.3. Biochemical analysis

Samples were thawed and centrifuged at 1750 × g for 10 min at room temperature, and the supernatant was immediately analyzed on BT1500 (Biotecnica, Rome, Italy) automated chemistry analyzer. On board were used Gesan's reagents (Italy) for all biochemical

parameters tested: alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, lactate dehydrogenase (LDH), creatine-kinase (CK), alkaline phosphatase (ALP), creatinine, urea, amylase, gamma-glutamyl transferase (γ-GT), triglycerides, cholesterol, total proteins, albumin, glucose, magnesium, potassium, sodium, calcium and phosphorus, to except for lipase (Biotecnica, Rome, Italy) and chloride (Biotecnica, Rome, Italy), while globulins were calculated as the difference between total protein and albumin. Osmolarity was calculated according to the following formula: $1.86 \times (Na + K) + 1.15 \times$ glucose + urea $+1.2 \times$ ethanol $+14$.

2.4. Statistical analysis

Data were analyzed for normality by Kolmogorof-Smirnof test and then submitted to: 1) Anova for the comparison of the amniotic and allantoic biochemical composition between normal and pathologic puppies; 2) Ancova mixed model in which, limited to fetal fluids biochemical parameters recorded in normal puppies, the type of fluid (amniotic or allantoic), the breed body size (small or large) and the newborn gender (male or female) were considered as fixed factors, and the maternal parity and Apgar score were considered as covariates; 3) the Spearman correlation test was used to verify possible correlations between the two fluids biochemical parameters in amniotic and allantoic fluids; 4) the coefficients of variation (CV) within litters and among litters were evaluated for each parameter in the amniotic and allantoic fluids.

3. Results

3.1. Clinical findings

From the 24 bitches, 1.5–9 years old, 1–4 parity, with litter size ranging between 2 and 7 puppies, a total of 76 newborn puppies was obtained. Seventy puppies (92.1%) were normal developed and weighted, healthy and viable, and therefore considered as "normal", while six (7.9%) were less viable and underweighted ($n = 5$), or malformed ($n = 1$), and considered as "pathologic". No born dead puppy was recorded. All those six pathologic puppies died within 24 h of birth, despite the provided reanimation and neonatal cares. Within the group of the 70 normal puppies, 33 were males and 37 females, and according to breed body size, 30 belonged to large and 40 to small breeds. All of them were alive at 24 h and at 7 days of age.

3.2. Fetal fluids analysis

Because of the possible loss of allantoic fluid occurring during the procedures of fetal bags opening, only 52 allantoic fluid samples were collected, while all the 76 amniotic samples were collected, stored and analyzed. Among the 52 allantoic fluid samples, 46 belonged to normal puppies and 6 to the pathologic ones. The amniotic ($n = 70$) and allantoic ($n = 46$) biochemical composition in the 70 normal newborn puppies, and the amniotic ($n = 6$) and allantoic ($n = 6$) biochemical composition in the 6 pathologic newborns were reported in Table 1, in which the normal newborn puppies were sub-grouped according to breed body size (small sized $n = 40$, large sized $n = 30$) and newborn gender (males $n = 33$, females $n = 37$). Data were expressed as mean ± SE, and (min-max) for each parameter.

Due to the small number of pathologic newborns, the statistical analysis failed to detect significant differences in fetal fluids biochemical composition between the normal and the pathologic newborns.

Maternal parity played a significant negative effect on amniotic and allantoic CK ($p < .05$), LDH ($p < .01$), and glucose ($p < .0001$), and a positive effect on amniotic and allantoic chloride ($p < .05$).

The Spearman correlation test evidenced a positive correlation between the two fetal fluids for albumin ($R = +0.60$, $p < .001$), phosphorus ($R = +0.59$, $p < .001$), glucose ($R = +0.93$, $p < .0001$), AST ($R = +0.53$, $p < .05$), and a negative correlation between the two fetal fluids for triglycerides ($R = -0.38$, $p < .05$).

The CV, used to verify the possible variability of each parameter within and among litters, showed a wide range (0–145.3 for amniotic and 0.1–145.9 for allantoic fluid) of variability within litter, and a less pronounced variability among litters (3.2–77.4 for amniotic and 14.4–71.3 for allantoic fluid).

4. Discussion

To the authors knowledge, this study describes, for the first time, the biochemical composition of both amniotic and allantoic fluids collected during elective CS in dogs at term of a full monitored normal pregnancy in at term, normal, healthy, viable dogs and preliminary data about pathologic littermates. The present study results, therefore, deserve interest because they add some basic information about dogs perinatology and represent a first step for the further comparison of both normal and pathologic fetal fluids composition. Despite the discrepancy between the number of "normal" and "pathologic" newborns, a first comparison was done between the fetal fluids characteristics in normal and pathologic puppies. However, as expected, the statistical analysis failed to detect significant differences between the two groups, due to the small number of pathologic puppies (6/76), that could have affected the statistical evaluation. In the present study, the 7.9% ratio of perimortality, at puppy level, is in agreement with the 8% rate reported by Tonnessen et al. [2]; therefore data from the present study can be considered representative of the common practice field conditions.

The apparent differences between fetal fluids composition in normal and pathologic puppies mainly regard some amniotic components, such as LDH, creatinine, UREA, γ-GT and, at a less extent, cholesterol and AST. For those parameters the maximum values in the pathologic puppies exceed the maximum values of the normal puppies (Table 1). Because all the 6 pathologic puppies died within the first day postpartum, these apparently different data seems to be of practical interest and need further, more detailed, investigations, to define whether or not they can provide suitable information about the risk for early neonatal mortality, and also to clarify whether they could represent causes or effects for the risk of a newborn puppy to die soon after birth.

When the statistical analysis was applied to the 70 normal puppies, marked differences between the two fluids for many parameters were detected, suggesting a different source and mechanism of production and accumulation of the two fluids, in dogs.

In detail, except for AST, triglycerides, cholesterol, albumin, amylase, glucose, and osmolarity, all the parameters were significantly different between the two fluids, although the Spearman correlation test showed a positive, from moderate to very strong, correlation between amniotic and allantoic albumin, phosphorus, glucose and AST, and a weak negative correlation between amniotic and allantoic triglycerides.

Because of the wide range of CV within litters, and, at a less extent, also among litters, for most of the studied parameters, indicative of a strong inter-individual variation, the possible effect played by some parameters such as maternal parity, breed body size, newborn gender and Apgar score, was statistically investigated.

According to breed body size, the differences observed about amniotic amylase, cholesterol, and allantoic calcium and glucose

Table 1
Amniotic (n = 70) and allantoic (n = 46) biochemical composition in the 70 normal newborn puppies, and amniotic (n = 6) and allantoic (n = 6) biochemical composition in the six pathologic newborns. The 70 normal newborn puppies were sub-grouped according to breed body size (small sized n = 40, large sized n = 30) and newborn gender (males n = 33, females n = 37). Data were expressed as mean ± SE, and (min-max) for each parameter.

	Normal puppies (n = 70)	Pathologic puppies (n = 6)	Normal Small sized (n = 40)	Normal Large sized (n = 30)	Normal Males (n = 33)	Normal Females (n = 37)
ALT (U/L)						
Amniotic fluid	1.10 ± 0.11[A]	1 ± 0.25	1.0 ± 0.11	1.2 ± 0.17	0.9 ± 0.14	1.3 ± 0.17
	(0.0–3.8)	(0.0–1.9)	(0.0–2.4)	(0.0–3.8)	(0.0–2.7)	(0.2–3.8)
Allantoic fluid	2.5 ± 0.80[B]	2.1 ± 0.04	2.9 ± 1.20	2.0 ± 1.10	2.8 ± 1.03	1.7 ± 0.39
	(0.4–9.6)	(2.1–2.2)	(1.0–7.7)	(0.4–9.6)	(0.4–9.6)	(0.6–2.2)
AST (U/L)						
Amniotic fluid	9.4 ± 0.58	10.2 ± 1.85	10.8 ± 0.77	8.7 ± 1.09	9.5 ± 0.80	9.5 ± 0.85
	(2.3–21.7)	(5.0–21.7)	(3.2–21.7)	(2.3–19.0)	(3.2–19.0)	(2.3–21.7)
Allantoic fluid	11.4 ± 1.16	16.4 ± 5.75	14.2 ± 1.68	10.5 ± 1.58	11.7 ± 1.33	13.4 ± 2.22
	(2.5–27.7)	(8.9–27.7)	(4.3–27.7)	(2.5–23.0)	(2.5–23.0)	(5.3–27.7)
TOTAL BILIRUBIN (mg/dl)						
Amniotic fluid	0.2 ± 0.02[a]	0.2 ± 0.05	0.2 ± 0.03	0.2 ± 0.02	0.2 ± 0.02	0.2 ± 0.03
	(0.0–0.7)	(0.0–0.4)	(0.0–0.7)	(0.0–0.5)	(0.0–0.4)	(0.0–0.7)
Allantoic fluid	0.6 ± 0.05[b]	0.4 ± 0.15	0.6 ± 0.06	0.5 ± 0.06	0.5 ± 0.05	0.6 ± 0.09
	(0.0–1.03)	(0.1–0.8)	(0.3–1.0)	(0.1–1.0)	(0.0–1.03)	(0.3–1.0)
LDH (U/L)						
Amniotic fluid	29.8 ± 2.80[a]	54.8 ± 20.61	28.2 ± 3.67	35.8 ± 6.91	36.3 ± 3.65	29.8 ± 4.29
	(2.5–173.8)	(12.8–173.9)	(4.7–63.7)	(2.5–173.9)	(4.7–173.8)	(2.5–90.8)
Allantoic fluid	116.6 ± 16.62[b]	78.9 ± 24.83	156.9 ± 34.69	88.5 ± 15.57	124.5 ± 20.64	75.8 ± 30.26
	(0.5–323.4)	(54–103.7)	(9.5–323.4)	(0.5–191.3)	(0.5–323.4)	(0.6–191.3)
CK (U/L)						
Amniotic fluid	3.2 ± 0.41[a]	2.5 ± 0.61	3.0 ± 0.74	3.3 ± 0.60	3.0 ± 0.69	3.1 ± 0.43
	(0.1–15.0)	(0.5–4.6)	(0.1–9.2)	(0.1–15.0)	(0.1–15.0)	(0.1–9.2)
Allantoic fluid	10.3 ± 1.72[b]	5.2 ± 0.82	15.1 ± 3.52	7.0 ± 1.33	10.8 ± 2.07	6.5 ± 2.36
	(0–34.8)	(4.4–6.1)	(1.2–34.8)	(0–19.9)	(1.2–34.8)	(0–12.6)
ALP (U/L)						
Amniotic fluid	126.0 ± 6.76[a]	122.4 ± 16.86	136.6 ± 11.08	117.2 ± 8.50	117.7 ± 8.99	133.1 ± 10.08
	(37.1–244.9)	(58.0–211.0)	(37.1–229.2)	(50.9–244.9)	(37.1–244.9)	(50.5–229.2)
Allantoic fluid	25.7 ± 3.29[b]	29.8 ± 8.21	30.2 ± 5.96	23.2 ± 3.71	24.8 ± 3.91	29.9 ± 6.05
	(2.2–81.6)	(22.0–38.0)	(8.5–81.6)	(2.2–59.0)	(2.2–81.6)	(9.3–59.0)
CREATININE (mg/dl)						
Amniotic fluid	2.8 ± 0.15[a]	3.3 ± 1.00	3.1 ± 0.57	2.8 ± 0.23	2.8 ± 0.28	3.1 ± 0.16
	(1.0–10.5)	(1.4–10.5)	(1.3–10.5)	(1.0–6.2)	(1.0–6.2)	(1.3–10.5)
Allantoic fluid	28.3 ± 2.33[b]	20.4 ± 6.86	33.7 ± 1.80	23.2 ± 3.39	26.7 ± 2.77	29.5 ± 4.48
	(1.3–43.9)	(4.6–37.6)	(16.8–41.4)	(1.3–43.9)	(1.3–43.9)	(3.3–41.8)
UREA (mg/dl)						
Amniotic fluid	40.7 ± 2.03[a]	46.1 ± 6.63	43.0 ± 3.29	41 ± 3.12	41.2 ± 3.35	41.7 ± 2.55
	(16.9–81.9)	(24.6–81.8)	(16.9–65.9)	(17.5–81.8)	(16.9–81.9)	(17.5–66.0)
Allantoic fluid	74.6 ± 7.43[b]	94.3 ± 20.95	73.8 ± 10.63	78.8 ± 10.50	75.4 ± 9.16	80.1 ± 10.82
	(23.3–186.8)	(62.6–151.2)	(24.4–163.4)	(23.3–186.9)	(23.4–186.8)	(29.5–134.5)
AMYLASE (U/L)						
Amniotic fluid	40.0 ± 3.01	28.0 ± 5.25	30.3 ± 11.24[x]	44.2 ± 5.08[y]	38.8 ± 4.35	37.8 ± 4.26
	(7.0–98.0)	(10.4–60.0)	(7.0–92.0)	(17.0–98.0)	(7.0–98.0)	(10.0–92.0)
Allantoic fluid	33.2 ± 1.90	31.8 ± 0.15	36.2 ± 2.69	30.6 ± 2.79	33.6 ± 2.03	32 ± 4.78
	(13.3–54.9)	(15.0–52.0)	(19–51.5)	(13.3–54.9)	(13.3–50.9)	(15.3–54.9)
LIPASE (U/L)						
Amniotic fluid	5.8 ± 0.25[a]	6.9 ± 0.66	5.7 ± 0.65	6.1 ± 0.45	6.2 ± 0.35	5.7 ± 0.35
	(3.5–10.0)	(3.6–9.4)	(3.9–8.9)	(3.5–10.0)	(3.9–10.0)	(3.5–9.5)
Allantoic fluid	11.6 ± 0.99[b]	9.6 ± 2.89	14.0 ± 1.37	9.5 ± 1.25	10.5 ± 1.15	13.8 ± 1.88
	(3.2–22.0)	(6.2–15.3)	(6.9–22.0)	(3.2–19.7)	(3.2–19.7)	(9.7–22.0)
γ-GT (U/L)						
Amniotic fluid	3.7 ± 0.24[a]	11.4 ± 5.35	4.1 ± 0.65	4.6 ± 0.35	5.0 ± 0.31	4.0 ± 0.38
	(0.5–37.2)	(2.6–37.2)	(0.5–12.6)	(1.3–37.2)	(0.5–37.2)	(1.2–12.6)
Allantoic fluid	59.8 ± 8.00[b]	48.9 ± 23.01	63.5 ± 9.29	55.6 ± 12.28	52.7 ± 8.25	80.4 ± 21.80
	(2.1–150.5)	(8.3–88.0)	(9.1–141.9)	(2.1–150.5)	(2.1–150.1)	(2.2–148.3)
TRIGLYCERIDES (mg/dl)						
Amniotic fluid	13.7 ± 2.31	13.3 ± 4.81	15.4 ± 3.88	13.3 ± 3.03	11.5 ± 2.33	15.7 ± 3.94
	(0–73.3)	(0–43.2)	(1.7–58.0)	(0–73.3)	(1.2–39.6)	(0–73.3)
Allantoic fluid	17.5 ± 3.28	20.7 ± 9.78	18.6 ± 3.45	17.3 ± 5.09	15.4 ± 3.26	25.7 ± 10.14
	(2.6–75.7)	(6.0–48.4)	(4.9–48.4)	(2.6–75.7)	(2.6–66.1)	(4.1–75.7)
CHOLESTEROL (mg/dl)						
Amniotic fluid	3.3 ± 0.41	5.4 ± 2.77	3.0 ± 0.89[x]	3.9 ± 0.91[y]	2.9 ± 0.55	4.0 ± 0.59
	(0.1–11.6)	(0.6–11.6)	(0.4–11.6)	(0.1–9.6)	(0.2–8.6)	(0.4–11)
Allantoic fluid	3.7 ± 0.56	5.6 ± 2.57	3.5 ± 0.42[x]	4.3 ± 0.90[y]	3.5 ± 0.70	4.9 ± 0.99
	(0–13.9)	(0.8–12.8)	(0.4–12.8)	(0–13.9)	(0.4–13.9)	(0–12.8)
TOTAL PROTEINS (g/L)						
Amniotic fluid	1.5 ± 0.18[a]	1.9 ± 0.05	1.8 ± 0.24	1.4 ± 0.25	1.3 ± 0.19	1.9 ± 0.29
	(0.1–6.9)	(0.1–4.6)	(0.4–4.6)	(0.1–6.9)	(0.2–4.5)	(0.1–6.9)
Allantoic fluid	5.2 ± 0.51[b]	3.6 ± 0.62	6.2 ± 0.70	4.5 ± 0.68	4.9 ± 0.59	5.7 ± 1.07
	(0.2–9.5)	(2.6–4.7)	(2.4–9.4)	(0.2–9.5)	(0.2–9.5)	(0.5–9.5)

Table 1 (*continued*)

	Normal puppies (n = 70)	Pathologic puppies (n = 6)	Normal Small sized (n = 40)	Normal Large sized (n = 30)	Normal Males (n = 33)	Normal Females (n = 37)
ALBUMIN (g/L)						
Amniotic fluid	0.8 ± 0.05 (0.3–2.2)	1.1 ± 0.18 (0.5–2.2)	0.9 ± 0.05 (0.4–1.5)	0.9 ± 0.07 (0.3–2.2)	0.9 ± 0.05 (0.4–2.2)	0.8 ± 0.07 (0.3–2.1)
Allantoic fluid	0.9 ± 0.05 (0.3–1.7)	1.5 ± 0.14 (1.2.1.7)	1.1 ± 0.09 (0.4–1.7)	0.8 ± 0.06 (0.3–1.3)	0.9 ± 0.06 (0.4–1.3)	0.9 ± 0.10 (0.3–1.7)
GLOBULINS (g/L)						
Amniotic fluid	1.0 ± 0.19[a] (0.1–4.4)	2.4 ± 0.66 (1.5–3.7)	1.6 ± 0.32 (0.2–4.1)	0.8 ± 0.33 (0.1–4.4)	1.1 ± 0.30 (0.1–4.1)	1.2 ± 0.24 (0.1–4.4)
Allantoic fluid	5.3 ± 0.46[b] (1.0–9.6)	2.2 ± 0.48 (1.4–3)	5.3 ± 0.76 (1.0–9.6)	4.7 ± 0.57 (1.2–9.1)	4.8 ± 0.54 (1.0–9.6)	5.5 ± 0.92 (3.0–9.1)
GLUCOSE (mg/dl)						
Amniotic fluid	24.5 ± 1.38 (2.1–51.5)	18.3 ± 4.44 (2.0–38.0)	23.2 ± 2.24 (5.8–51.5)	24.9 ± 1.78 (8.7–46.1)	28.1 ± 2.08 (8.7–51.5)	19.7 ± 1.71 (2.1–44.2)
Allantoic fluid	26.6 ± 2.22 (1.5–47.4)	27.9 ± 6.49 (9.5–40.0)	24.4 ± 3.63[X] (1.5–47.3)	28.7 ± 2.78[Y] (11.0–45.0)	28.6 ± 2.57 (1.5–47.4)	21.1 ± 3.96 (9.5–41.4)
MAGNESIUM (mg/dl)						
Amniotic fluid	1.1 ± 0.05[a] (0.4–2.1)	0.9 ± 0.12 (0.4–1.5)	1.1 ± 0.08 (0.5–2.1)	1.0 ± 0.06 (0.4–1.9)	1 ± 0.06 (0.4–1.9)	1.1 ± 0.07 (0.4–2.1)
Allantoic fluid	5.9 ± 0.46[b] (0.9–8.5)	4.6 ± 1.68 (1.1–8.2)	7.0 ± 0.60 (0.9–8.5)	4.8 ± 0.62 (0.9–8.1)	5.5 ± 0.55 (0.9–8.5)	6.6 ± 0.78 (1.1–8.3)
POTASSIUM (mmol/L)						
Amniotic fluid	5.5 ± 0.16[a] (2.9–9.3)	5.7 ± 0.58 (3.1–8.5)	5.5 ± 0.19 (3.8–8.5)	5.6 ± 0.29 (2.9–9.3)	5.5 ± 0.27 (3.8–9.3)	5.6 ± 0.20 (2.9–9.2)
Allantoic fluid	23.1 ± 1.60[b] (3.1–30.9)	21.2 ± 5.42 (7.4–30.9)	25.8 ± 1.99 (7.3–30.9)	20.7 ± 2.35 (3.1–30.9)	21.6 ± 1.88 (3.1–30.9)	26.9 ± 2.91 (6.7–30.9)
CHLORIDE (mmol/L)						
Amniotic fluid	117.2 ± 0.96[a] (83.9–125.5)	114.0 ± 3.82 (87–124.0)	115.1 ± 1.14 (87.0–124.8)	117.8 ± 1.39 (83.9–125.5)	118.3 ± 0.86 (108.3–125.0)	115.4 ± 1.65 (84.0–125.5)
Allantoic fluid	82.4 ± 4.85[b] (34.2–120.6)	86.9 ± 14.43 (53.0–113.0)	68.4 ± 7.52 (34.2–112.1)	94.0 ± 5.49 (41.6–120.6)	84.3 ± 5.57 (34.3–120.6)	78.7 ± 10.50 (35.0–119.5)
SODIUM (mmol/L)						
Amniotic fluid	142.8 ± 1.74[a] (100.5–169.9)	142.4 ± 4.21 (113.8–153.3)	139.5 ± 3.40 (100.5–169.9)	145.0 ± 1.82 (113.6–159.0)	145.0 ± 2.01 (109.3–159.5)	140.7 ± 2.80 (100.5–159.9)
Allantoic fluid	76.9 ± 7.70[b] (1.4–147.7)	93.5 ± 22.10 (34.1–128.4)	61.5 ± 12.34 (1.4–140.9)	91.9 ± 9.24 (18.5–147.7)	82.7 ± 9.27 (12.5–147.7)	66.9 ± 13.04 (1.4–118.4)
CALCIUM (mg/dl)						
Amniotic fluid	4.3 ± 0.18[a] (1.9–9.3)	4.1 ± 0.82 (1.9–8.9)	4.2 ± 0.41 (2.3–8.9)	4.3 ± 0.24 (1.9–9.3)	4.1 ± 0.30 (1.9–9.3)	4.4 ± 0.21 (2–8.9)
Allantoic fluid	10.7 ± 1.17[b] (2.2–23.4)	9.5 ± 3.68 (2.2–19.7)	15.5 ± 1.78[x] (5.9–23.4)	7.2 ± 1.00[y] (2.2–21.3)	11.2 ± 1.42 (2.2–23.4)	9.8 ± 1.62 (4.8–19.7)
PHOSPHORUS (mg/dl)						
Amniotic fluid	5.2 ± 0.54[a] (1.6–18.9)	4.0 ± 0.54 (2.3–5.3)	4.3 ± 0.39 (1.6–13.3)	5.7 ± 0.85 (2.2–18.9)	4.0 ± 0.43 (1.6–10)	6.0 ± 0.88 (1.6–18.9)
Allantoic fluid	12.3 ± 2.85[b] (2.6–53.9)	29.6 ± 10.41 (3.4–54.2)	9.6 ± 0.86 (2.6–53.3)	17.3 ± 4.49 (2.7–54.0)	9.9 ± 2.61[Z] (2.6–53.9)	27.5 ± 8.87[W] (2.7–53.2)
OSMOLARITY (mOsm/l)						
Amniotic fluid	298.5 ± 3.00 (108.0–330.6)	304.1 ± 5.95 (273.6–322.8)	293.5 ± 5.21 (221.6–330.6)	302.3 ± 3.45 (235.1–330.6)	294.7 ± 6.93 (108.0–330.6)	296.2 ± 4.90 (221.6–327.2)
Allantoic fluid	223.5 ± 10.87 (97.8–364.9)	244.7 ± 33.73 (153.6–308.9)	224.4 ± 20.88 (109.5–364.9)	248.4 ± 12.06 (113.9–364.9)	226.9 ± 14.19 (109.5–362.9)	224.6 ± 15.18 (97.8–309.4)

[A,B] denote differences between amniotic and allantoic fluid with $p < .01$.
[a,b] denote differences between amniotic and allantoic fluid with $p < .0001$.
[X,Y] denote differences between small and large sized normal fetuses with $p < .01$.
[x,y] denote differences between small and large sized normal fetuses with $p < .05$.
[Z,W] denote differences between genders in normal fetuses with $p < .05$.

could be dependent on the body size or may indeed reflect a breed specific variation. In fact, breed specific variations were recently reported on serum creatinine, amylase, lipase, ALT and phosphorus by Chang et al. [9], highlighting a potential genetic contribution of biochemical traits in the dog species. However the breed-specific differences was not assessed in the present study, because of small number of subjects for such statistical analysis.

About the negative (CK, glucose, LDH), or positive (chloride) correlation between some amniotic and allantoic parameters with increasing maternal parity, it is difficult to hypothesize a scientific explanation of this results. Unfortunately, in human studies the effect of parity was not considered as a factor affecting the amniotic fluid biochemical composition.

The difference in allantoic phosphorus concentrations in female as compared to male puppies, was the sole significant finding influenced by the newborn gender. Although Chang et al. [9], recently reported differences between sexes for dogs serum total proteins, globulin, creatinine, amylase and lipase, the authors are not aware of studies investigating the effect of newborn gender in the fetal fluids biochemical composition in other species. None effect played by the Apgar score was detected in the group of normal newborns (Apgar score ≥ 7). This result is not surprising because a possible effect of the Apgar score could be supposed for the newborns scored <7, but the number of those puppies (n = 5) was too low to allow a statistical analysis.

4.1. Liver enzymes

The statistical analysis showed a significant higher ALT activity in allantoic as compared to amniotic fluid. This finding, different from the results reported by Fresno et al. [10] in the cat, is difficult to explain and simply indicates a non specific cell damage, higher in the allantoic than in the amniotic compartment. The lack of significant differences in AST activity at term, was also previously reported for the cat by Fresno et al. [10]. When the AST and ALT values were compared to data reported for feline fetal fluid collected at term, amniotic AST and ALT were rather similar, while allantoic AST and ALT resulted lower in dogs than in cats. Fetal fluids AST, and especially ALT activity, were very lower when compared to newborn puppies [11,12] or adult dogs serum levels [13]. This finding is very interesting because it could be the indicative of the newborn dog liver immaturity, even if, being those parameters indicative of non specific cell damage, this hypothesis must be considered cautiously. Total bilirubin concentration and LDH activity were also significantly higher in allantoic fluid when compared to amniotic fluid, similarly to data reported in the cat at term by Fresno et al. [10], suggesting, once more, a possible contribution of the gastrointestinal tract to the final allantoic composition. Amniotic total bilirubin was similar to that reported for the cat, while the allantoic total bilirubin observed in dogs was one fourth lower in comparison to the cat, but higher than data reported for adult dogs serum [13]. LDH activity was lower in both fluids when compared to data reported for the cat at term by Fresno et al. [10], but this difference between the two species is difficult to be explained, even if it could be the result of a liver immaturity. The very wide range falls however within the range reported for adult dog serum levels by ZhanKui et al. [14]. Amniotic and allantoic LDH activity was negatively influenced by the increased maternal parity ($p < .01$), even if this finding is difficult to be explained.

4.2. Muscular enzymes

The CK values in both fetal fluids were similar to those reported by Fresno et al. [10], in the cat, but strikingly lower when compared to newborn puppies [11,12] or adult dogs serum [15] levels. A significant higher CK activity was found in allantoic than in the amniotic fluid, but a suitable explanation for this finding is difficult.

Amniotic and allantoic CK activity was lower as maternal parity increased ($p < .05$), but also for this finding an explanatory reason is difficult.

Differently to the cat, in the present study a significant difference between amniotic and allantoic ALP activity was found. This difference, although difficult to explain, could be addressed to the fetal poly-systemic contribution to the amniotic composition and the pronounced fetal proliferative cellular activity. The amniotic highest value was very similar to the mean value reported for the adult dog serum [15], but lower in comparison to newborn puppies serum, especially at birth [13,14].

4.3. Kidney enzymes

As expected, and similar to results reported for the cat, creatinine and urea concentrations were higher in the allantoic than in the amniotic fluid. These results seems to suggest that, in the dog, the majour source for the allantoic fluid are fetal urines, as previously reported for humans by Oliveira et al. [16], and could be a function of the fetal kidney filtration. However, in the present study, the amniotic creatinine and urea concentrations were a bit higher than the mean value reported for the human normal pregnancy between 36 and 42 weeks by Oliveira et al. [16]. The present study creatinine and urea fetal fluids values were similar to those reported for the cat, but extremely higher when compared to adult and newborn dogs serum [11–13].

4.4. Pancreas/gastrointestinal enzymes

Although amylase activity was not different between the two fluids, amniotic amylase activity was significantly higher in large sized breed dogs respect to small sized breeds. This finding could be tempting explained by the cumulative differences about the amylase synthesis, catabolism and excretion that could be, at least partly, influenced genetically. Values are lower in comparison to those reported for the cat fetal fluids, especially when allantoic activity was compared. If compared to newborn puppies or adult serum levels, fetal fluids amylase activity was found to be about one tenth lower, indicating a possible immature activity of the pancreatic enzymes in dogs at term of the intrauterine stage of development. When lipase activity was concerned, it resulted higher in allantoic than in amniotic fluid, differently to what reported for the cat by Fresno et al. [10]. However, when compared to newborn puppies or adult serum, fetal fluids lipase values were very low.

4.5. Bile duct enzymes

Gamma-glutamyl transferase values were very similar to those reported for the cat [10], but in the present study a higher activity was found in the allantoic in comparison to the amniotic fluid. This higher values of γ-GT in allantoic fluid, difficult to explain, could suggests the contribute of the gastrointestinal tract in the final allantoic fluid composition.

4.6. Lipid metabolism enzymes

Both triglycerides and cholesterol concentrations were not significantly different between the two fluids, similarly to what reported for the cat [10]; however lower amniotic and allantoic cholesterol concentrations were found in small sized as compared to large sized breeds. In serum adult dogs, Chang et al. [9] recently reported different serum cholesterol concentrations according to breeds grouping. Therefore a certain influence of the breed could be also supposed to influence the fetal cholesterol metabolism, resulting in lower cholesterol fetal fluids concentrations in small sized dogs. Fetal fluids cholesterol concentrations resulted about one hundred times lower than values reported for the newborn puppies [11] and adult dogs [13] serum.

4.7. Proteins, glucose

Total proteins and globulins concentrations were higher in allantoic fluid in comparison to the amniotic counterpart, similar to findings reported for the cat by Fresno et al. [10]. No differences were found between the two fluids albumin concentrations, in agreement with data reported for the cat [10]. Amniotic albumin was higher than the mean value reported for the human normal pregnancy at 36–42 weeks by Oliveira et al. [16].

Likely, glucose concentrations were not different between the two fluids, as previously reported in the cat. However, in the present study higher allantoic glucose concentrations were found in large sized than in small sized dogs, as well as a negative relation between increasing parity and glucose amniotic and allantoic concentrations. Amniotic glucose concentration was a bit higher than the value reported for the human normal pregnancy between 36 and 42 weeks by Oliveira et al. [16]. Fetal fluids total proteins, globulins and albumin were very lower when compared to newborn puppies [11] or adult dogs [13] serum values, while fetal

fluids glucose concentrations were about one third lower when compared to adult dogs serum values [13], and almost 6 times lower when compared to newborn puppies serum values [11].

4.8. Electrolytes

Although Fresno et al. [10] did not find differences between the amniotic and allantoic magnesium, potassium, chloride, sodium, calcium and phosphorus concentrations in the cat at term, in the present study magnesium, potassium and phosphorus concentrations resulted higher in allantoic than in amniotic fluid, while chloride, sodium, and calcium concentrations were higher in the amniotic fluid. In addition, allantoic calcium concentration was higher in small sized dogs, allantoic chloride concentration increased parallel to maternal parity, and allantoic phosphorus concentration was higher in females when compared to male newborns. This last result was in contrast to the reported higher serum phosphorus concentration in 6 months old male dogs [17], or with the absence of significant differences between sexes in adult dogs reported by Chang et al. [9]. When data were compared to the amniotic electrolytes composition reported by Oliveira et al. [16] in humans normal pregnancy between 36 and 42 weeks, sodium concentrations were very similar, while potassium concentrations were slightly higher, calcium concentrations slightly lower, and phosphorous concentrations definitively higher.

When compared to newborn puppies [11] and adult dogs serum values [13], amniotic calcium and even more allantoic calcium concentrations were higher. Amniotic potassium and magnesium concentrations were very similar to newborn puppies and adult dogs serum values, and allantoic values were higher. Amniotic chloride and sodium concentrations were very similar to data reported for newborn puppies and adult dogs, while allantoic chloride and sodium concentrations were lower. Phosphorus resulted higher in both amniotic and allantoic fluids in comparison to the values reported for the newborn puppies and adult dogs serum. The low concentration of fetal fluids sodium, significantly lower in the allantoic fluid, is in agreement with data reported in humans, in which the reabsorption of sodium from the fetal kidney is considered as about 85–95% of the filtered load, indicating that, at term, the fetal kidney has a very efficient reabsorption ability [16].

It remains difficult to explain the high fetal fluids concentrations of phosphorus.

4.9. Osmolarity

Osmolarity was not different between the two fluids and also between normal and pathologic puppies. No effect played by maternal or fetal parameters on amniotic and allantoic fluids collected by the normal puppies was detected. Osmolarity of amniotic fluid was very similar to the one reported for at term amniotic fluid in cat [10] and woman [16], while allantoic osmolarity was a bit lower in comparison to the value reported for the cat by Fresno et al. [10].

5. Conclusions

In conclusion, the present study provided first data about the biochemical composition of canine fetal fluids collected from at term, normal developed, healthy and viable dogs and preliminary data about a small group of pathologic littermates.

In the normal conditions, beside few correlations, the results showed many differences between the amniotic and the allantoic biochemical composition, suggesting that diverse sources could concur to the final composition of each fluid. For some parameters an influence of breed body size, maternal parity and newborn gender was found.

A wide variability within and among litters was found for both amniotic and allantoic biochemical parameters, indicating a strong inter-individual variability.

Further investigations are needed for addressing the origin and meaning of each fetal fluid biochemical composition in the dog and also to assess possible differences in fetal fluids biochemical composition between normal and pathologic puppies, providing potential markers for the quick identification of newborns at risk, that need special surveillance and cares, immediately after birth.

Conflicts of interest

The authors have no competing interests.

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References

[1] Veronesi MC. Assessment of canine neonatal viability – the Apgar score. Reprod Domest Anim 2016;51:46–50.
[2] Tønnessen R, Borge KS, Nødtvedt A, Indrebø A. Canine perinatal mortality: a cohort study of 224 breeds. Theriogenology 2012;77:1788–801.
[3] Gill MA. Perinatal and late neonatal mortality in the dog. Doctor of Philosophy Thesis. University of Sidney; 2011.
[4] Bowden RST, Hodgman SFJ, Hime JM. Neo-natal mortality in dogs. In: Proceedings 17th world veterinary congress, hannover; 1963. p. 1009–13.
[5] Vassalo FG, Simões CR, Sudano MJ, Prestes NC, Lopes MD, Chiacchio SB, et al. Topics in the routine assessment of newborn puppy viability. Top Companion Anim Med 2015;30:16–21.
[6] Brace RA, Wlodek ME, Cock ML, Harding R. Swallowing of lung liquid and amniotic fluid by the ovine fetus under normoxic and hypoxic conditions. Am J Obstet Gynecol 1994;171:764–70.
[7] Underwood MA, Gilbert WM, Sherman MP. Amniotic fluid: not just fetal urine anymore. J Perinatol 2005;25:341–8.
[8] Meloni T, Comin A, Rota A, Peric T, Contri A, Veronesi MC. IGF-I and NEFA concentrations in fetal fluids of term pregnancy dogs. Theriogenology 2014;8:1307–11.
[9] Chang YM, Hadox E, Sziadovitis B, Garden OA. Serum biochemical phenotypes in the domestic dog. PLos One 2016;11(e), e0149650.
[10] Fresno L, Rodriguez-Gil JE, Rigau T, Pastor J, Riviera del Alamo MM. Modulation of the biochemical composition of amniotic and allantoic fluids as a control mechanism of feline foetal development. Placenta 2012;33:522–7.
[11] Rørtveit R, Saevik BK, Eggertsdóttir AV, Skancke E, Lingaas F, Thoresen SI, et al. Age-related changes in hematologic and serum biochemical variables in dogs aged 16-60 days. Vet Clin Pathol 2015;44:47–57.
[12] Rosset E, Rannou B, Casseleux G, Chalvet-Monfray K, Buff S. Age-related changes in biochemical and hematologic variables in Borzoi and Beagle puppies from birth to 8 weeks. Vet Clin Pathol 2012;41:272–82.
[13] Dunlop MM, Sanchez-Vazquez MJ, Freeman KP, Gibson G, Sacchini F, Lewis F. Determination of serum biochemistry reference intervals in a large sample of adult greyhounds. J Small Anim Pract 2011;52:4–10.
[14] ZhanKui Y, JieXin L, XiuTing L, DeGui L. Serum biochemical indicators of hepatobiliary function in dogs following prolonged anaesthesia with sevoflurane or isoflurane. Vet Anaesth Analg 2012;39:296–300.
[15] Schäfers A, Meierhans S, Sauter-Louis C, Hartmann K, Hirschberger J. Reference values for haematological and clinical-chemical parameters in the dog. Tierarztl Prax Ausg K Kleintiere Heimtiere 2013;41:163–72.
[16] Oliveira FR, Barros EG, Magalhães JA. Biochemical profile of amniotic fluid for the assessment of fetal and renal development. Braz J Med Biol Res 2002;35:215–22.
[17] Choi SY, Hwang JS, Kim IH, Hwang DY, Kang HG. Basic data on the hematology, serum biochemistry, urology, and organ weights of beagle dogs. Lab Anim Res 2011;27:283–91.

CHAPTER 12

Use of hairs and nails as a new matrices for non-invasive hormonal and metabolic perinatal investigations

USE OF HAIRS AND NAILS AS A NEW MATRICES FOR NON-INVASIVE HORMONAL AND METABOLIC PERINATAL INVESTIGATIONS

BACKGROUND

In human medicine researches about neonatal physiology were historically performed using blood or cord blood samples (Gitau et al, 2001), but the invasiveness of blood sampling and the disadvantage of the single point measurement prevent its use in perinatology. Furthermore, in puppies the possibility of blood sampling is limited by the invasiveness of sampling and by the impossibility of serial sampling due to the limited blood collectable volume (Munnich l, 2008; Veronesi et al, 2013).

THE NEED FOR NON-INVASIVE MATRICES

For these reasons, alternative and more suitable matrices for analysis must be considered. In human-beings the analysis of amniotic fluid composition received, in the last decades, a strong scientific interest, so that many aspects were fully investigated, and markers for the correct management of both the pregnant woman and of fetuses/newborns were detected. In particular, some amniotic parameters near term of pregnancy were proved to be useful for the early recognition of fetal well-being disturbances and neonatal outcome in humans (Wiberg-Itzel et al, 2014), but also in other animal species (Koski and Ferguson, 1992; Pirrone et al, 2012).

Recently, some studies reported the use of hairs for the cortisol measurement in humans (Raul et al, 2004; Sauvé et al, 2007; Kirschbaum et al, 2009; D'Anna-Hernandez et al, 2011) and animals (Davenport et al, 2006; Accorsi et al, 2008; Macbeth et al, 2010; González-de-la-Vara Mdel et al, 2011; Comin et al, 2012), proving the usefulness of this biological matrix for the non-invasive, and long time-frame retrospective picture of previous hormone accumulation assessment.

Interestingly, the availability of easily accessible biomarkers for fetal and neonatal biology investigation could allow to enhance the knowledge about both the late intrauterine and early neonatal dog physiology. When the study of a long-term hormonal changes is requested, such as during a certain fetal or neonatal period of development, the hair (Bryan et al, 2013) represents the best matrix for the retrospective, cumulative assessment.

Several studies reported the use of hair for long-term cortisol accumulation assessment in humans and other animal species.

Even though the hair represent a useful matrix for hormone measurement in newborns, in the foetus the hair start to grow in late pregnancy, so that its measurement provides information limited to the last period of intrauterine development. Similarly to the hair, also nails analysis allows the assessment of hormones accumulated over a long time period, passively diffused from capillaries to the matrix of nails and incorporated into the keratin along nail growth. Strengths of using nails as matrix for hormonal analysis includes therefore its potential as retrospective parameter of intrauterine events. In addition, as compared to the hair, the nails start to growth early in gestation (around the 8th week of gestation, in humans), so that the cumulative information cover a longer window of time in comparison to the hair (de Berker et al, 2007; Palmeri et al, 2000; Ben Khelil et al, 2011).

Recently, the activity of hypothalamic-pituitary-adrenal axis activity were investigated measuring both cortisol and dehydroepiandrosterone (DHEA) in fingernails of adult humans, collected without invasiveness and stored at room temperature, (Min-Soo et al, 2010; Warnock et al, 2010; D'Anna-Hernandez et al, 2011), while DHEA and dehydroepiandrosterone sulfate (DHEAS) were measured in nails of infants (Tegethoff et al, 2011), opening new potential perspectives for the retrospective investigation of perinatal biology in several clinical fields, and for the early detection of endocrine disorders and enzyme defects.

The study of hairs for the detection of endogenous corticosteroids was done for the first time on wilde hyrax where a positive correlation was found between cortisol concentration in hairs and social rank of the animal (Koren et al 2002). Starting from this study investigations on corticosteroids hair levels were done in different animal species. Cortisol levels were investigated in hairs of macaque, primates, chimpanzees, polar bear and grizzly (Davenport et al, 2006; Fourie e Bernstein, 2011; Bechsoft et al 2011; Yamanashi et al, 2013; Bryan et al, 2013). Cortisol levels were measured also in domestic animals, such as the bovine, swine and canine species. In cows, in fact, a study evaluated cortisol concentration in relation to the presence of pathologies such as endomentritis, mastitis and laminitis (Comin et al, 2013). In the sow cortisol levels were related to animal welfare and reproductive attitude (Bacci et al, 2014). In dogs the first study was done by Accorsi et al (2008), and evidenced a positive correlation between cortisol concentrations in hairs and feces, but, at the same time, authors found some problems related to the poor knowledge about hair physiology and cortisol accumulation in hairs in the dog species. Another study demonstrated a positive correlation between cortisol concentration in hairs and saliva, and reported low cortisol levels in black hairs as compared to light ones (Bennet e Hayssen, 2010). A recent study by Mack and Fokidis (2017) detected the cortisol concentration in hairs and nails of dogs and found a positive correlation between cortisol concentration in nails and hairs. In this study cortisol concentration did not differ from female to male dogs, but all dogs were spayed or neutered; cortisol concentration did not differ also in relation to the age of dogs, to the breed or to the body size.

In veterinary neonatology hairs were used in newborn foals to detect cortisol levels at birth and at 30 and 60 days of age, and a decrease of cortisol levels after birth and during the first 60 days of age was evidenced (Comin et al, 2012).

Also claws were used for the detection of cortisol levels in animal species. In particular Comin et al (2014) investigated cortisol concentration in claws of calves, finding higher cortisol concentrations in claws collected from 0 to 30 days of age in comparison to claws collected at 31-60 and 61-120 days of age.

HAIR AND NAIL AS MATRICES FOR THE STUDY OF CANINE PERINATOLOGY

The availability of easily accessible biomarkers for fetal and neonatal biology investigation is promising also for the study of late intrauterine and early neonatal dog physiology. As well as for human neonates, also for puppies, non invasive and simple techniques of investigation are mandatory. Furthermore, when the study of a long-term period hormonal changes is requested, such as during a certain fetal or neonatal periods, the nails (Tegethoff et al, 2011) and hairs (Veronesi et al, 2015) represent the best matrix for retrospective, cumulative investigations. As reported for human newborns, also for puppies the collection of nails by clipping provides a simple, non-invasive and repeatable method for hormones profiles investigations.

In dog fetuses, the hair starts to grow late during gestation, in fact it appears at around 45 days of gestation, so that the analysis of hormonal accumulation in the hair collected at birth will provide information about the last intrauterine stages of development. Another valuable matrix, providing information about hormonal accumulation, is represented by the nails (Warnock et al 2010; Maidana et al, 2013). Even if there are no references regarding the exact time of nails appearance in dog fetuses, a study reported that, to the authors observation, full visible nails are detectable from 30 days of gestation (Veronesi et al, 2015). An additional advantage of nails and hair as matrices of study, is represented by the fact that they can be collected without invasiveness in newborn dogs and stored at room temperature until analysis.

In dogs neonatology there is only a study investigating hormonal levels in nails and hairs of newborn puppies. This recent study investigated cortisol levels in hairs and nails collected from 165 spontaneously dead purebred puppies, classified according to newborn gender, breed body size and age at death. This study reported the usefulness of hairs and nails as a new matrices for perinatal hormonal investigations also in dogs, and reported a strong positive correlation between cortisol concentrations in nails and hairs, similarly to what subsequently reported by Mack and Fokidis (2017). The authors investigated the possible influence of newborn breed body size and gender on cortisol levels, but did not found significant results. In that study only newborn age seemed to have an influence on cortisol concentrations in hairs and nails, in fact cortisol levels were higher in premature puppies as compared to term born-dead puppies and puppies dead during the first 30 days of age (Veronesi et al, 2015).

HPA and DHEA-DHEAS

The HPA axis is a key system that is essential in fetal maturation, development and homeostasis, and it also play a role in the preparation for the survival of newborns (Mastorakos and Ilias, 2003). In humans the fetal pituitary matures early during gestation and the activity of fetal HPA axis begins at mid-gestation. The fetal adrenal gland is composed by different zones: the outer definitive zone, the inner fetal zone and, between these, the transition zone, that has intermediate characteristics. The fetal zone rapidly disappears after birth and its involution seems to be related to the gestational age (Ishimoto and Jaffe, 2011).

The transition zone cells synthesize cortisol, in fact they are analogous of the zona fasciculata cells in the adult adrenal cortex; DHEA and its sulfatated derivate DHEAS, are produced by cells of the fetal zone and are considered the major steroids produced by the fetus itself (Mesiano and Jaffe 1997; Pasqualini, 2005; Ishimoto and Jaffe, 2011;). In humans, DHEAS production seems to begin

at 8-10 weeks of gestation, with an increase in the production during the second and third trimester of pregnancy. Transient cortisol production occurs early in gestation (7-10 weeks of gestation), then the cortisol biosynthesis is suppressed until late gestation, when the cortisol production escalates (Ishimoto and Jaffe; 2011).

The time of birth, in humans, is regulated by a mechanism that synchronizes fetal maturation with the initiation of parturition processes. In humans and also in other animals, such as the sheep, glucocorticoids are essential for cell differentiation and organ maturation in the fetus (Challis et al, 2000; Challis et al, 2001; Ishimoto e Jaffe, 2011). Near term, the fetal HPA axis produces cortisol and DHEA/DHEAS with an important role in the regulation of parturition. More specifically, the placenta-derived corticotrophin-releasing hormone (CRH) increases and stimulate directly the fetal HPA that produces cortisol and DHEA/DHEAS. The cortisol, in turn, stimulates the production of placental CRH. Cortisol also promotes the fetal multi-organ maturation (lung) and stimulates the production of prostaglandins necessary for the parturition process. DHEA/DHEAS are converted by the placenta to estrogens, promoting the initiation of parturition (Ishimoto and Jaffe, 2011).

In humans DHEA and DHEAS are the most abundant circulating steroid hormones; in particular large amount of these hormons are produced during the fetal development and, after birth, their production decreases rapidly and remains low for several years (Abdulmaged et al, 2011).

DHEA is reported as a selective glucocorticoid receptor antagonist and plays an important role in the developing brain in the fetus, protecting the neurons against glucocorticoid-induced neurotoxicity; DHEA also modulates the effects of corticosteroids in the brain. In human neonates low levels of plasma DHEA relative to cortisol have been correlated with behavioral pathologies in adults (depression, schizophrenia), revealing the importance of DHEA in the fetal and neonatal brain development (Quinn et al, 2013). DHEA plays a role not only in the nervous system; in fact,

at a tissue level, DHEA was reported as the main adrenal steroid with anti-glucocorticoid properties in humans (El-Eshmawy et al, 2011). Additionally, DHEA was proved to reverse the pulmonary hypertension in both adult humans and infant rats (Dumas de la Roque et al, 2013), suggesting a significant role in the management of such important disease also in human newborns.

The activity of human fetal HPA axis was historically investigated by fetal blood or cord blood analysis or by amniotic fluid analysis (Bergman et al, 2010; Gitau et al, 2011; Kajantie et al, 2014), although those methods showed important limitations. In fact, as fetal body fluids are poorly accessible, sampling procedures are invasive. Furthermore, all these matrices provide a single-point measurement (Taylor et al, 2000).

For all these reasons, recently, the activity of HPA axis in the fetus was investigated using new matrices such as the hairs and nails. In fact the collection of these matrices is non-invasive and easy to be performed, and they provide information about hormonal accumulation during a long time-frame (Teghetoff et al, 2011).

CHAPTER 13

References

REFERENCES

Accorsi PA, Carloni E, Valsecchi P, Viggiani R, Gamberoni M, Tamanini C, Seren E. (2008) Cortisol determination in hair and faeces from domestic cats and dogs. Gen Comp Endocrinol 155, 398-402.

Bacci ML, Nannoni E, Govoni N, Scorrano F, Zannoni A, Forni M, Martelli G, Sardi L. (2014) Hair cortisol determination in sows in two consecutive reproductive cycles. Reprod Biol 14, 218-223.

Bechshøft TØ, Sonne C, Dietz R, Born EW, Novak MA, Henchey E, Meyer JS. (2011) Cortisol levels in hair of East Greenland polar bears. Sci Total Environ 409, 831-834.

Ben Khelil M, Tegethoff M, Meinlschmidt G, Jamey C, Ludes B, Raul JS. (2011) Simultaneous measurement of endogenous cortisol, cortisone, dehydroepiandrosterone, and dehydroepiandrosterone sulfate in nails by use of UPLC-MS-MS. Anal Bioanal Chem 401(4), 1153-1162.

Bennett A, Hayssen V. (2010) Measuring cortisol in hair and saliva from dogs: coat color and pigment differences. Domest Anim Endocrin 39, 171-180.

Bergman K, Sarkar P, Glover V, O'Connor TG. (2010) Maternal prenatal cortisol and infant cognitive development: moderation by infant-mother attachment. Biol Psychiat 67, 1026-1032.

Bryan HM, Adams AG, Invik RM, Wynne-Edwards KE, Smits JEG. (2013) Hair as a meaningful measure of baseline cortisol levels over time in dogs. J Am Assoc Lab Anim Sci 52(2), 189-196.

Bryan HM, Darimont CT, Paquet PC, Wynne-Edwards KE, Smits JEG. (2013) Stress and reproductive hormones in grizzly bears reflect nutritional benefits and social consequences of a salmon foraging niche. PLoS ONE 8, e80537.

Challis JR, Sloboda D, Matthews SG, Holloway A, Alfaidy N, Patel FA, Whittle W, Fraser M, Moss TJ, Newnham J. (2001) The fetal placental hypothalamic-pituitary-adrenal (HPA) axis, parturition and postnatal health. Mol Cell Endocrinol 185:135–144

Challis JRG, Matthews SG, Gibb W, Lye SJ. (2000) Endocrine and paracrine regulation of birth at term and preterm. Endocr Rev 21:514–550

Comin A, Peric T, Corazzin M, Veronesi MC, Meloni T, Zufferli V, Cornacchia G, Prandi A. (2013) Hair cortisol as a marker of hypothalamic-pituitary-adrenal axis activation in Friesian dairy cows clinically or physiologically compromised. Livestock Science 152, 36-41.

Comin A, Peric T, Magrin L, Corazzin M, Cornacchia G, Prandi A. (2014) Study of progesterone and cortisol concentrations in the Italian Friesian claw. Journal of Dairy Science 97, 5491-5496.

Comin A, Veronesi MC, Montillo M, Faustini M, Valentini S, Cairoli F, Prandi A. (2012) Hair cortisol levels as a retrospective marker of hypothalamic-pituitary-adrenal axis activity in horse foals. Vet J 194(1), 131-132.

D'Anna-Hernandez KL, Ross RG, Natvig CL, Laudenslager ML. (2011) Hair cortisol levels as a retrospective marker of hypothalamic-pituitary axis activity throughout pregnancy: Comparison to salivary cortisol. Physiol Behav 104, 348-353.

Davenport MD, Tiefenbacher S, Lutz CK, Novak MA, Meyer JS. (2006) Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. Gen Comp Endocrinol 147, 255-261.

De Berker DAR, Andre J, Baran R. (2007) Nail biology and nail science. Inter J Cosm Sci 29, 241-275.

Dumas de la Roque E, Quignard JF, Ducret T, Dahan D, Courtois A, Begueret H, Marthan R, Savineau JP. (2013) Beneficial effect of dehydroepiandrosterone on pulmonary hypertension in a

rodent model of pulmonary hypertension in infants. Pediatr Res. 2013 Aug;74(2):163-9. doi: 10.1038/pr.2013.73. Epub 2013 May 6.

El-Eshmawy MM, Hegazy A, EL-Baiomy AA. (2011) Relatinship between IGF-1 and Cortisol/DHEA-S ratio in adult men with diabetic metabolic syndrome versus non-diabetic metabolic syndrome. J Endocrino Metab 2011; 1:188-95.

Fourie NH, Bernstein RM. (2011) Hair cortisol levels track phylogenetic and age related differences in hypothalamic-pituitary-adrenal (HPA) axis activity in non-human primates. Gen Comp Endocr 174, 150-155.

Gitau R, Menson E, Pickles V, Fisk NM, Glover V, MacLachlan N. (2001) Umbilical cortisol levels as an indicator of the fetal stress response to assisted vaginal delivery. Eur J Obstet Gynecol Reprod Biol 2001; 98: 14-7.

González-de-la-Vara Mdel R, Valdez RA, Lemus-Ramirez V, Vázquez-Chagoyán JC, Villa-Godoy A, Romano MC. (2011) Effects of adrenocorticotropic hormone challenge and age on hair cortisol concentrations in dairy cattle. Can J Vet Res. 2011 Jul;75(3):216-21.

Ishimoto H, Jaffe RB. (2010) Development and function of the human fetal adrenal cortex: a key component in the feto-placental unit. Endocr Rev. 2011 Jun;32(3):317-55. doi: 10.1210/er.2010-0001. Epub 2010 Nov 4. Review.

Kajantie E, Raivio T, Jänne OA, Hovi P, Dunkel L, Andersson S. (2004) Circulating glucocorticoid bioactivity in the preterm newborn after antenatal betamethasone treatment. J Clin Endocrinol Metab 2004; 89:3999-4003.

Kirschbaum C, Tietze A, Skoluda N, Dettenborn L. (2009) Hair as a retrospective calendar of cortisol production-increased cortisol incorporation into hair in the third trimester of pregnancy. Psychoneuroendocr 34, 32-37.

Koren L, Mokady O, Karaskov T, Klein J, Koren G, Geffen E. (2002) A novel method using hair for determining hormonal levels in wildlife. Anim Behav 2002; 63:403-6.

Koski KG, Ferguson MA. (1992). Amniotic fluid composition responds to changes in maternal dietary carbohydrate and is related to metabolic status in term fetal rats. J Nutr, 122, 385-392.

Macbeth BJ, Cattet MRL, Stenhouse GB, Gibeau ML, Janz DM. (2010) Hair cortisol concentration as a noninvasive measure of long-term stress in free-ranging grizzly bears (Ursus arctos): considerations with implications for other wildlife. Can J Zool 88, 935-949.

Mack Z, Fokidis HB. (2017) A novel method for assessing chronic cortisol concentrations in dogs using the nail as a source. Domest Anim Endocrinol. 2017 Apr;59:53-57. doi: 10.1016/j.domaniend.2016.11.003. Epub 2016 Nov 17.

Maidana P, Bruno OD, Mesch V. (2013) A critical analysis of cortisol measurements: an update. Medicina (B Aires) 73(6), 579-84.

Mastorakos G, Ilias I. (2003) Maternal and fetal hypothalamic-pituitary-adrenal axes during pregnancy and postpartum. Annals of the New York Academy of Sciences 997, 136-149.

Mesiano S, Jaffe RB. (1997) Developmental and functional biology of the primate fetal adrenal cortex. Endocrine Reviews 18, 378-403.

Min-Soo K, Young-Jin L, Ryun-Sup A. (2010) Day-to-day differences in cortisol levels and molar cortisol-to-DHEA ratios among working individuals. Yonsei Med J 2010; 51:212-8.

Münnich A. (2008) The pathological newborn in small animals: the neonate is not a small adult. Vet Res Commun. 2008 Sep;32 Suppl 1:S81-5. doi: 10.1007/s11259-008-9096-0.

Palmeri A, Pichini S, Pacifici R, Zuccaro P, Lopez A. (2000) Drugs in nails: physiology, pharmacokinetics and forensic toxicology. Clin Pharmacokinet 38(2), 95-110.

Pasqualini JR. (2005) Enzymes involved in the formation and transformation of steroid hormones in the fetal and placental compartments. J Steroid Biochem Mol Biol. 2005 Dec;97(5):401-15. Epub 2005 Oct 3. Review.

Pirrone A, Mariella J, Gentilini F, Castagnetti C. (2012) Amniotic fluid and blood lactate concentrations in mares and foals in the early postpartum period. Theriogenology 78(6), 1182-1189.

Quinn TA, Ratnayake U, Dickinson H, Nguyen TH, McIntosh M, Castillo-Melendez M, Conley AJ, Walker DW. (2013) Ontogeny of the adrenal gland in the spiny mouse, with particular reference to production of the steroids cortisol and dehydroepiandrosterone. Endocrinol. 2013 Mar;154(3):1190-201. doi: 10.1210/en.2012-1953. Epub 2013 Jan 25.

Raul J, Cirimele V, Ludes B, Kintz P. (2004) Detection of physiological concentrations of cortisol and cortisone in human hair. Clin Biochem 37(12), 1105-11.

Sauvé B, Koren G, Walsh G, Tokmakejian S, VanUum S. (2007) Measurement of cortisol in human hair as a biomarker of systemic exposure. Clin Invest Med 30, E183-E191.

Tegethoff M, Raul JS, Jamey C, Khelil MB, Ludes B, Meinlschmidt G. (2011) Dehydroepiandrosterone in nails of infants: a potential biomarker of intrauterine responses to maternal stress. Biol Psychol 2011; 87:414-20.

Traish AM, Kang HP, Saad F, Guay AT. (2011) Dehydroepiandrosterone (DHEA)--a precursor steroid or an active hormone in human physiology. J Sex Med. 2011 Nov;8(11):2960-82; quiz 2983. doi: 10.1111/j.1743-6109.2011.02523.x.

Veronesi MC, Comin A, Meloni T, Faustini M, Rota A, Prandi A. (2015) Coat and claws as new matrices for noninvasive long-term cortisol assessment in dogs from birth up to 30 days of age. Theriogenology 2015; 84:791-6.

Veronesi MC. (2013): Cenni di fisiologia neonatale. In: Veronesi, M.C., Castagnetti, C., Taverne, M.A.M. (eds) Neonatologia veterinaria. EdiSES, Napoli, pp. 11-30.

Warnock F, McElwee K, Seo RJ, McIsaac S, Seim D, Ramirez-Aponte T, Macritchie KA, Young AH. (2010) Measuring cortisol and DHEA in fingernails: a pilot study. Neuropsychiatr Dis Treat 6, 1-7.

Wiberg-Itzel E, Pembe AB, Wray S, Wihlbäck AC, Darj E, Hoesli I, Åkerud H. (2014) Level of lactate in amniotic fluid and its relation to the use of oxytocin and adverse neonatal outcome. Acta Obstetr et Gyn Scandinavica, 93, 80-85.

Yamanashi Y, Morimura N, Mori Y, Hayashi M, Suzuki J. (2013) Cortisol analysis of hair of captive chimpanzees (Pan troglodytes). Gen Comp Endocrinol 2013; 194:55-63.

CHAPTER 14

Aim of the second step of the PhD project

AIM OF THE SECOND STEP OF THE PhD PROJECT

To date little is known about the hypothalamic-pituitary-adrenal axis activity in canine fetuses and newborns. In order to improve knowledge about the perinatal physiology in dogs and in particular on the activity of the hypothalamic-pituitary adrenal axis, the second part of the PhD project focused on the use of hairs and nails as non-invasive matrices for the measurement of DHEA and DHEAS in newborn puppies.

CHAPTER 15

Hair dehydroepiandrosterone (DHEA) concentrations in newborn dogs

HAIR DEHYDROEPIANDROSTERONE (DHEA) CONCENTRATIONS IN NEWBORN DOGS

The last intrauterine fetal stage of development and the neonatal period represent the most challenging phases for the mammals offspring. Toward the end of pregnancy, the hypothalamic-pituitary-adrenal axis (HPA) of the fetus becomes a key system regulating several physiologic processes, such as the fetal multi-organs final maturation (Bolt et al, 2001), the stress response (Tegethoff et al, 2011), and the triggering of parturition. After birth, and during the neonatal period, the newborn undergoes several physiologic and metabolic changes necessary for survival and health, the HPA system keeps a role in the neonatal adaptation to the extra-uterine life (Thorburn et al, 1972; Fowden, 1995). In response to the adrenocorticotrophic hormone (ACTH), the adrenals produce cortisol and dehydroepiandrosterone (DHEA), characterized by opposite effects (Buhimschi et al, 2008). DHEA was reported as the main steroid produced by the fetal adrenals with antiglucocorticoids proprieties, especially at a tissue level (El-Eshmawy et al, 2011). In fetuses and newborns the activity of the hypothalamic-pituitary-adrenal axis was historically investigated by fetal blood or cord blood analysis (Dumas de la Roque et al, 2013; Kajantie et al, 2004), but these methods were limited by the invasiveness of sampling and by the single-point measurement. More recently cortisol were measured in hairs of humans and animals proving the usefulness of this matrix for non-invasive, retrospective and long time-frame hormonal accumulation analysis. In particular, in the study of neonatology, cortisol was measured in hairs of newborn preterm babies to assess the effect of hospitalization in comparison with healthy newborns (Yamada et al, 2007) founding elevated levels of cortisol in hairs of preterm babies in comparison with healty newborns. Hair cortisol was also measured in newborn horse foals from birth to 60 days of age, and a cortisol decline, probably due to the neonatal adaptation to the extra uterine environment, reported (Comin et al, 2012). In dogs, hair cortisol was investigated in adult dogs (Accorsi et al, 2008; Bryan et al, 2013) and, recently, also in spontaneously dead

newborn puppies (Veronesi et al, 2015). In the last mentioned study, the authors found higher cortisol concentrations in hairs and also in nails of premature newborn puppies in comparison to fresh term born-dead puppies and puppies dead in the first 30 days of age. However the fetal or maternal origin of the cortisol accumulated in the newborns hair was not proved.

The investigation of DHEA in hairs of newborn puppies deserves interest in the study of canine perinatology and could better elucidate the role of the fetus itself in hormonal hair accumulation around the time of birth.

To date, however, little is known about DHEA accumulation in hairs of dogs, and DHEA concentrations were never investigated in hairs of newborn puppies. Therefore the present study aimed to investigate DHEA concentration in hairs of spontaneously dead purebred puppies and the possible influence on hormonal hairs accumulation of newborn age, gender and breed body size.

REFERENCES

Accorsi PA, Carloni E, Valsecchi P, Viggiani R, Gamberoni M, Tamanini C, Seren E. (2008) Cortisol determination in hair and faeces from domestic cats and dogs. Gen Comp Endocrinol 155, 398-402.

Bolt RJ, van Weissenbruch MM, Lafeber HN, Delemarre-van de Waal HA. (2001) Glucocorticoids and lung development in the fetus and preterm infant. Pediatr Pulmonol 2001;32:76-91.

Bryan HM, Adams AG, Invik RM, Wynne-Edwards KE, Smits JEG. (2013) Hair as a meaningful measure of baseline cortisol levels over time in dogs. Journal of the American Association for Laboratory Animal Science 52, 189-196.

Buhimschi CS, Turan OM, Funai EF, Azpurua H, Bahtiyar MO, Turan S, Zhao G, Dulay A, Bhandari V, Copel JA, Buhimschi IA. (2008) Fetal adrenal gland volume and cortisol/dehydroepiandrosterone sulfate ratio in inflammation-associated preterm birth. Obstet Gynecol 2008; 111:715-22.

Comin A, Veronesi MC, Montillo M, Faustini M, Valentini S, Cairoli F, Prandi A. (2012) Hair cortisol level as a retrospective marker of hypothalamic-pituitary-adrenal axis activity in horse foals. Vet J 194, 131-132.

Dumas de la Roque E, Quignard JF, Ducret T, Dahan D, Courtois A, Begueret H, Marthan R, Savineau JP. (2013) Beneficial effect of dehydroepiandrosterone on pulmonary hypertension in a rodent model of pulmonary hypertension in infants. Pediatr Res 2013; 74:163-9.

El-Eshmawy MM, Hegazy A, EL-Baiomy AA. (2011) Relatinship between IGF-1 and Cortisol/DHEA-S ratio in adult men with diabetic metabolic syndrome versus non-diabetic metabolic syndrome. J Endocrino Metab 2011; 1:188-95.

Fowden AL. (1995) Endocrine regulation of fetal growth. Reprod Fertil Dev 1995; 7:351-63.

Kajantie E, Raivio T, Jänne OA, Hovi P, Dunkel L, Andersson S. (2004) Circulating glucocorticoid bioactivity in the preterm newborn after antenatal betamethasone treatment. J Clin Endocrinol Metab 2004; 89:3999-4003.

Tegethoff M, Raul JS, Jamey C, Khelil MB, Ludes B, Meinlschmidt G. (2011) Dehydroepiandrosterone in nails of infants: a potential biomarker of intrauterine responses to maternal stress. Biol Psychol 2011; 87:414-20.

Thorburn GD, Nicol DH, Bassett JM, Shutt DA, Cox RI. (1972) Parturition in the goat and sheep: changes in corticosteroids, progesterone, oestrogens and prostaglandin F. J Reprod Fertil Suppl 1972; 16: 61-84.

Veronesi MC, Comin A, Meloni T, Faustini M, Rota A, Prandi A. (2015) Coat and claws as new matrices for noninvasive long-term cortisol assessment in dogs from birth up to 30 days of age. Theriogenology 2015; 84:791-6.

Yamada J, Stevens S, de Silva N, Gibbins S, Beyene J, Taddio A, et al. (2007) Hair cortisol as a potential biologic marker of chronic stress in hospitalized neonates. Neonatology 92, 42-49.

CHAPTER 16

Hair dehydroepiandrosterone (DHEA) concentrations in newborn dogs

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Hair dehydroepiandrosterone (DHEA) concentrations in newborn dogs

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Introduction and aim. The last intrauterine foetal stage of development and the neonatal period represent the most challenging phases for the mammals' offspring. During this time-frame the hypothalamic-pituitary-adrenal axis (HPA) is a key system regulating several physiologic processes, such as the foetal multi-organs final maturation, the response to stress, and after birth the HPA keeps a role in the neonatal adaptation to the extra-uterine life. Apart of cortisol, in response to the adrenocorticotrophic hormone, the adrenals produce dehydroepiandrosterone (DHEA). Some studies in humans and other animal species demonstrated that the foetal pituitary-adrenal axis secretes DHEA under activation from maternal cortisol. Thus, DHEA measured at birth in newborns could be considered as a marker of offspring HPA activity, under the maternal influence. HPA activity, in foetus as well as in newborns, was previously investigated by foetal blood or cord blood analysis, but these methods were limited due to the invasiveness of sampling and to the single point measurements. Non invasive, and long time-frame retrospective hormonal levels analysis were performed in the hair of humans and animals, but not in the hair of newborn puppies, and to the authors knowledge DHEA was never investigated in puppies,. This study was aimed to assess DHEA concentrations in the hair of newborn puppies, and to evaluate the possible influence of newborn age and gender, and breed size on DHEA concentrations.

Materials and methods. The study enrolled 116 spontaneously dead purebred puppies, 55 females and 61 males. According to maternal body weight, puppies were classified as belonging to small size breeds (bodyweight ≤10 kg) (N = 48), or to a merged class of medium and large size breeds (bodyweight >10 kg) (N = 68). Based on the age at death, all newborns were grouped in three classes of age, as follows: 1) premature: when puppies were delivered between 2 and 1 weeks before the expected date of whelping, estimated by ultrasonographic parturition date prediction [1] (N=19); 2) fresh, term born dead puppies (puppies delivered dead at the predicted term of pregnancy and puppies born alive at term, but dead within 24 hours since birth (N=79); 3) puppies dead between 1 and 30 days of age (N=28). Hair samples were collected from each puppy by shaving, and stored at room temperature until analysis by RIA [2].

Results. In the hair of the 116 puppies, the overall DHEA concentration was 46.8±14.8 pg/mg. According to newborn gender, DHEA levels were 48.6±15.66 pg/mg in females vs 45.1±13.73 pg/mg in males, without significant differences. In relation to breed size, DHEA levels were 45.5±13.61 pg/mg in small size breed puppies compared to 47.8±15.61 pg/mg in medium-large size breed puppies, with no significant differences. According to age at death, DHEA hair concentrations in premature puppies (52.5±15.12 pg/mg) were significant higher ($p<0.05$) than in puppies dead between 1 and 30 days after birth (44.5±17.78 pg/mg), but similar to fresh term born dead puppies (46.2±16.5 pg/mg).

Conclusions. This study demonstrated that DHEA is quantifiable in the hair of newborn dogs, and interesting for non invasive, long time-frame foetal and neonatal studies also in this species. The results obtained from 116 newborns evidenced that only the newborn age influenced DHEA, while newborn gender or breed or body size did not. The effect of age is in agreement with different DHEA plasma concentrations related to age, in adult dogs, while the absence of significant influence of sex disagree with data reported for adult dogs [3], maybe because of the very young age of the studied puppies.

References

1) Beccaglia and Luvoni, J Small Anim Pract 2006;47:670-73. 2) Peric et al., Proc 2nd Dairycare Conference 2015;Cordoba,Spain:68. 3) Mongillo et al., Res Vet Sci 2014;96:33-8.

CHAPTER 17

Claws dehydroepiandrosterone and dehydroepiandrosterone sulfate concentrations in newborn dogs

CLAWS DEHYDROEPIANDROSTERONE AND DEHYDROEPIANDROSTERONE SULFATE CONCENTRATIONS IN NEWBORN DOGS

In the last intrauterine fetal stages of development the hypothalamic-pituitary-adrenal axis became a key system of the developing fetus that plays also an important role in the neonatal adaptation to extra-uterine life during the neonatal period. In fact the HPA axis keeps a role in several physiologic processes such as the fetal multi-organs maturation, the triggering of parturition and the response to stress (Bolt et al, 2001; Tegethoff et al, 2011), and, after birth, it still has a role in the neonatal adaptation to extra-uterine life (Thorburn et al, 1972; Fowden, 1995). In response to the adrenocorticotrophic hormone the adrenals produce cortisol and DHEA, the last one is then conveted into his transport form DHEAS, characterized by opposite effects (Buhimschi et al, 2008). In humans DHEA was reported as the main steroid produced by the fetus itself with anti-glucocorticoid effects (El-Eshmawy et al, 2011).

The activity of HPA axis was previously investigated in humans and animals by blood or cord blood analysis (de Fencl and Tulchinsky , 1975; Gitau et al, 2001), however these methods were limited by the invasiveness f sampling and by the single point measurement (Tegethoff et al, 2011). Recently some studies reported the use of hairs as an innovative matrix for the study of cortisol concentration in humans and animals. In addition hair samples collection is non-invasive and hairs analysis provides a retrospective and long time-frame information on hormonal (Gow et al, 2000; Sauvé et al, 2007; Accorsi et al, 2008; Comin et al, 2012). Another keratinized matrix with an homologous structure in comparison to the hairs are the nails (Warnock et al, 2010; Izawa et al, 2015), that can give, similarly, information about retrospective and long time-frame hormonal levels accumulation, as demonstrated in human studies (de Berker et al, 2007; Ben Khelil et al, 2011). In humans, DHEA and DHEAS were analyzed in nails of newborns in order to verify the influence of maternal stress during pregnancy on fetal stress biology (Tegethoff et al, 2011). Nails

were also used in veterinary neonatology to verify the cortisol accumulation in spontaneously dead purebred newborn dogs (Vereonesi et al, 2015).

REFERENCES

Accorsi PA, Carloni E, Valsecchi P, Viggiani R, Gamberoni M, Tamanini C, Seren E. (2008) Cortisol determination in hair and faeces from domestic cats and dogs. Gen Comp Endocrinol 155, 398-402.

Ben Khelil M, Tegethoff M, Meinlschmidt G, Jamey C, Ludes B, Raul JS. (2011) Simultaneous measurement of endogenous cortisol, cortisone, dehydroepiandrosterone, and dehydroepiandrosterone sulfate in nails by use of UPLC-MS-MS. Anal Bioanal Chem 401(4), 1153-1162.

Bolt RJ, van Weissenbruch MM, Lafeber HN, Delemarre-van de Waal HA. (2001) Glucocorticoids and lung development in the fetus and preterm infant. Pediatr Pulmonol 2001;32:76-91.

Buhimschi CS, Turan OM, Funai EF, Azpurua H, Bahtiyar MO, Turan S, Zhao G, Dulay A, Bhandari V, Copel JA, Buhimschi IA. (2008) Fetal adrenal gland volume and cortisol/dehydroepiandrosterone sulfate ratio in inflammation-associated preterm birth. Obstet Gynecol 2008; 111:715-22.

Comin A, Veronesi MC, Montillo M, Faustini M, Valentini S, Cairoli F, Prandi A. (2012) Hair cortisol level as a retrospective marker of hypothalamic-pituitary-adrenal axis activity in horse foals. Vet J 194, 131-132.

De Berker DAR, Andre J, Baran R. (2007) Nail biology and nail science. Inter J Cosm Sci 29, 241-275.

de Fencl M, Tulchinsky D. (1975) Total cortisol in amniotic fluid and fetal lung maturation. N Engl J Med 1975; 292:133-6.

El-Eshmawy MM, Hegazy A, EL-Baiomy AA. (2011) Relatinship between IGF-1 and Cortisol/DHEA-S ratio in adult men with diabetic metabolic syndrome versus non-diabetic metabolic syndrome. J Endocrino Metab 2011; 1:188-95.

Fowden AL. (1995) Endocrine regulation of fetal growth. Reprod Fertil Dev 1995; 7:351-63.

Gitau R, Menson E, Pickles V, Fisk NM, Glover V, MacLachlan N. (2001) Umbilical cortisol levels as an indicator of the fetal stress response to assisted vaginal delivery. Eur J Obstet Gynecol Reprod Biol 2001; 98: 14-7.

Gow R, Thomson S, Rieder M, Van Uum S, Koren G. (2010) An assessment of cortisol analysis in hair and its clinical applications. Forensic Sci Int 2010; 196:32-7.

Izawa S, Miki K, Tsuchiya M, Mitani T, Midorikawa T, Fuchu T, Komatsu T, Togo F. (2015) Cortisol level measurements in fingernails as a retrospective index of hormone production. Psychoneuroendocrinology (2015) 54, 24—30.

Sauvé B, Koren G, Walsh G, Tokmakejian S, Van Uum SH. (2007) Measurement of cortisol in human hair as a biomarker of systemic exposure. Clin Invest Med 2007; 30:E183-91.

Tegethoff M, Raul JS, Jamey C, Khelil MB, Ludes B, Meinlschmidt G. (2011) Dehydroepiandrosterone in nails of infants: a potential biomarker of intrauterine responses to maternal stress. Biol Psychol 2011; 87:414-20.

Thorburn GD, Nicol DH, Bassett JM, Shutt DA, Cox RI. (1972) Parturition in the goat and sheep: changes in corticosteroids, progesterone, oestrogens and prostaglandin F. J Reprod Fertil Suppl 1972; 16: 61-84.

Veronesi MC, Comin A, Meloni T, Faustini M, Rota A, Prandi A. (2015) Coat and claws as new matrices for noninvasive long-term cortisol assessment in dogs from birth up to 30 days of age. Theriogenology 2015; 84:791-6.

Warnock F, McElwee K, Seo RJ, McIsaac S, Seim D, Ramirez-Aponte T, Macritchie KA, Young AH. (2010) Measuring cortisol and DHEA in fingernails: a pilot study. Neuropsychiatr Dis Treat 6, 1-7.

CHAPTER 18

Claws dehydroepiandrosterone and dehydroepiandrosterone sulfate concentrations in newborn dogs

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P 22 | Effect of super weak static magnetic fields on reactive oxygen species generation in bovine spermatozoa

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The interaction of static magnetic fields (SMF) with biological objects is a rapidly growing field of investigation. Previous research showed that strong SMF influences somatic mammalian cells causing proinflammatory changes and increasing reactive oxygen species (ROS) production. On the other hand, super weak SMF (0.3 μT) affects proliferation and differentiation of skeletal muscle cells in the primary culture. Investigation of gametes' functioning in a shielded SMF is of great interest due to enhancing human presence in space, and agricultural animals are suitable models for the research. The aim of this study was to evaluate the effect of super weak SMF (0.3 μT) on ROS generation in bovine sperm. Fresh semen was obtained from Holstein bulls. A special camera (Russian Patent No. 2324989) was constructed for shielding down geomagnetic field. Semen was washed twice in Sp-TALP medium and then incubated for 4 h in a CO_2-incubator at 5% CO_2, 95% humidity and 38°C. To detect ROS production sperm cells ($12.5x10^6$ sperm/ml per sample) were supplemented with luminol (25 mM) and horse-radish peroxidase (11.52 U/ml). Chemiluminescence was monitored for 900 s. in a luminometer (Lum-5773) and the results expressed as integrated counts. Seven replicates were done in this study. There was a significant increase in the level of ROS generation in bovine spermatozoa incubated in the super weak SMF (678.9 ± 47.5 eV × s in experimental group vs. 490.7 ± 98.2 eV × s in control group, $p < 0.05$, Student's t-test).

P 23 | Claws dehydroepiandrosterone and dehydroepiandrosterone sulfate concentrations in newborn dogs

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DHEA and DHEA sulfate (DHEAS) in human newborns at birth are considered markers of fetal stress (Tegethoff et al. 2011, *Biol Psychol* 87:414–420). The hair and the claws could be useful, non invasive matrices for long time-frame retrospective hormonal analysis (Veronesi et al. 2015, *Theriogenology* 84:791–796). The aim of this study was to assess DHEA and DHEAS concentrations in the claws of newborn puppies, and to evaluate the possible influence of newborn age (premature, born dead, dead 1–10, 11–20, 21–30 days old), gender, and breed size on DHEA and DHEAS concentrations. The study, performed on 138 spontaneously dead purebred puppies, showed that the overall mean DHEA and DHEAS concentrations in claws were significantly different ($p < 0.05$) even if highly correlated ($p < 0.0001$) (28.8 ± 17.00 pg/mg and 36.5 ± 23.13 pg/mg, respectively). DHEAS levels were significantly higher ($p < 0.05$) in small as compared to large breeds (39.2 ± 25.45 pg/mg vs. 34.8 ± 21.51 pg/mg), and in premature vs. born dead and vs. 11–20 days old puppies (48.7 ± 28.63 pg/mg vs. 34.3 ± 20.89 and 29.9 ± 17.19 pg/mg, respectively) ($p < 0.05$). The effect of age on DHEAS levels in claws was previously reported also for DHEA hair concentrations in newborn puppies (Bolis et al. 2015, Proc EVSSAR 33:283–290). DHEA and DHEAs in claws of newborn puppies can be considered markers of perinatal stress, useful for research investigations and for future clinical application.

P 24 | A case of simultaneous pregnancy, pyometra and fetal mummification in a cat

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Cases of concurrent pregnancy and pyometra in cats are described, but additional presence of a mummified fetus is an extremely rare finding. A free-roaming, around 4-year-old, domestic short hair cat was presented with loss of appetite. Clinical examination revealed mild dyspnea and abnormalities on abdominal palpation. Complete blood count showed leukocytosis, while serum biochemistry was within normal limits. Thoracic and abdominal radiographs in DV and lateral projections were performed. There was one normal in size and shape, mineralized fetus and one with decreased bone density and irregularly located, multiple ossification centers. Abdominal ultrasound was made using a 6.5 MHz microconvex probe (Samsung). It revealed a properly developed, approximately 55-day-old fetus in one uterine horn and enlargement of the other horn, with thickened wall and fluid accumulation. Additionally, in the abdominal cavity there was a 36 × 24 mm mass of unknown origin, which contained focal mineralizations inside. No other abnormalities were noted in the abdomen. Ovariohysterectomy was elected, during which a free mummified fetus was found in the peritoneal cavity, between the intestinal loops. The fetus was not attached to any neighbouring organs. One of the uterine horns contained living fetus, while focal pyometra was present in the second. Uterine wall specimens were submitted for histopathological examination. The cat was treated with a standard dose of amoxicillin/clavulanic acid, recovered well and was set free 10 days after surgery.

P 25 | Fungal isolation from bovine milk samples in an Italian dairy farm: preliminary results

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CHAPTER 19

General discussion and conclusions

GENERAL DISCUSSION AND CONCLUSIONS

Because of the increasing interest in canine neonatology and the need of non invasive methods to study fetal and neonatal physiology, the present PhD project was aimed to indeep the knowledge about fetal fluids characteristics and some hormonal changes measured on other matrices collectable without invasiveness, such as the hair and the nails.

The first aim of the present thesis was to investigate some characteristics of amniotic and allantoic fluids belonging to normal, viable and well-developed newborn puppies, born at term of normal gestations, for the possible comparison with fetal fluids belonging to less viable, or diseased newborns, born by normal term gestations.

The first study investigated the cortisol concentrations in fetal fluids of small sized purebred dogs and the possible relation with newborn survival in the first 24 hours after birth. At the physiologic term of pregnancy cortisol concentrations were higher in allantoic fluid in comparison to amniotic fluid with a strong positive correlation in the cortisol concentrations between the two fluids, suggesting at least a concurrence of the fetus itself on the final cortisol accumulation in both fetal fluids. No significant correlation between cortisol fetal fluids concentrations and maternal parity, litter size and maternal body size were found, as well as no effect of neonatal gender was detected. An interesting finding was the significantly higher amniotic cortisol concentrations found in puppies dead 24 hours after birth in comparison to puppies alive at 24 hours of age, suggesting a possible prognostic role of cortisol concentration in amniotic fluid on newborn short-term survival. This last result need, however, to be confirmed by further studies on a large number of subjects in order to verify if cortisol fetal fluids concentrations measured at the time of birth could be used as a prognostic parameter for the short-term survival in newborn puppies.

The second study investigated the concentrations of uric acid, lactate, glucose and creatinine in amniotic fluid of small sized purebred newborn dogs born by elective cesarean section at term of normal pregnancies, in relation to newborn outcome and the possible effect played by maternal parity and newborn gender on uric acid, glucose, lactate and creatinine concentrations. In spite of the small number of puppies dead in the first 24 hours after birth in comparison with the number of normal puppies, the statistical analysis showed that glucose concentration was lower in puppies dead at 24 hours after birth in comparison with puppies alive at 7 days of age. The results, although deserves further investigations, seems to suggest that glucose could be considered as a possible marker useful to identify puppies that need special surveillance during the first 24 hours of age in order to reduce neonatal losses. When the statistical analysis was performed on fetal fluids belonging to normal puppies no significant difference on uric acid concentration were found in relation to maternal parity or newborn gender. Regarding amniotic glucose concentration a significant influence of newborn gender, but not of maternal parity, was found. Amniotic lactate concentration was higher in multiparous in comparison to primiparous bitches. Regarding creatinine no significant differences were found in relation to maternal parity or newborn gender. Further studies are needed in order to better investigate the possible differences between fetal fluids of normal and pathological puppies and the possible role of some metabolites in amniotic fluid as markers to detect puppies at risk of death in the first 24 hours after birth.

The third study investigated the biochemical composition of fetal fluids at term pregnancy in dogs. A comparison between fetal fluids characteristics of normal and pathologic puppies was done, but, the statistical analysis did not show significant results, due to the small number of pathologic puppies. When the statistical analysis was applied to the normal puppies, differences between the two fluids were found for many parameters, suggesting a different source and mechanism of production and accumulation of the two fluids in dogs. The study showed also the possible

influence of breed body size and of maternal parity and newborn gender on some parameters. Further studies are needed in order to better investigate possible differences between fetal fluids belonging to normal and to pathological puppies, and therefore to detect potential markers of fetal/neonatal diseases or for a quick identification of newborns at risk, that need special surveillance and cares, immediately after birth.

The last two studies focused on the possible use of hairs and nails as new matrices for the non-invasive, retrospective and long time-frame hormonal levels analysis. As already reported for cortisol, these studies focused on the activity of the hypothalamic-pituitary-adrenal axis activity in fetal and newborn dogs, by analyzing DHEA and DHEAS concentrations in hairs and nails of newborn puppies and assessing the possible relations between homonal levels and age at death, newborn gender and breed body size. Both the studies evidenced an influence of age at death on DHEA and DHEAS concentrations, in particular DHEA was higher in hairs of premature puppies in comparison to puppies dead between 1 and 30 days after birth, but similar to fresh term born dead puppies. Regarding nails, DHEA and DHEAS concentrations were significantly different in the overall concentration of DHEA and DHEAS in nails of newborn puppies, with a high correlation between the concentrations in the two matrices; DHEAS and not DHEA concentrations were significantly higher in small size breeds in comparison with large size breeds, while DHEAS was higher in premature puppies when compared to puppies born-dead or dead between 11 and 20 days of age. When the usefulness of these non-invasive matrices for the study of the dog perinatology was considered, the nails resulted more suitable in comparison to the hairs. In fact, in the perspective of using these matrices in alive newborn puppies, at present the relatively large amount of hair necessary for the analysis prevent its use on alive newborns.

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