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Comparation of Foetal Growth in Natural Mated and Vitrified/Warmed Ovine Embryos by Ultrasonographic Measurements

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Abstract: Ultrasonography has become the method of choice for monitoring gestation in veterinary obstetric. We provided a chronological description of the development of the ovine conceptus between day 30th and 100th, based on 3 different ultrasonographic morphometric foetal parameters: CRL (Crown-rump length), BPD (Biparietal diameter) and TL (Tibia length) and compared these data with ones registered during foetal growth of vitrified/warmed IVP embryos transferred into recipients ewes. Our results showed a regular increasing of the CRL and BPD whereas TL showed a regular increasing trend from day 54th to 89th, when it starts a faster raise until 100th day. No significant differences were observed between the parameters of NM (natural mating) group and V/W (Vitrified/Warmed embryos) group. On the other hand we could highlight an increase of the gestation period in the vitrified/warmed group and the absence of spontaneous lambing in 35%. Newborns derived after transfer of IVP vitrified embryos did not show any macroscopical alterations as well as the control group.

Key words: Ultrasonography · Foetal Development · Morphometric Foetal Parameters · IVP

INTRODUCTION

Ultrasonography, first used in veterinary medicine in 1980's, has become the method of choice for monitoring the gestation period in several domestic species.

The use of ultrasound in veterinary obstetric also allows to perform the foetal sex determination and can be an important clinical technique to observe the physiological and pathological events during foetal development. Previous studies on embryonic and foetal growth in domestic animals were carried out using animals sacrificed at various stages of gestation [1] or using radiographic technique [2].

Small ruminants pregnancy can be verified by ultrasonography as early as 20-22 days after mating using transrectal or even transcutaneous probes and various studies have been performed to analyze the foetal growth, physical integrity of the foetus and its normal anatomical and physiological development [3-7]. Interest on small ruminants foetal growth, particularly in sheep, has recently increased because ovine foetus is used as a model to study human foetal development. In fact BPD and other morphometric parameters, as well as birth weight, are comparable to those obtained in human foetus [8] in spite of a duration of pregnancy that is approximately half as long. Several parameters have been estimated by real time ultrasonography, measuring BPD and other two parameters: heart rate and the diameter of the body trunk [9].

Moreover the estimation of foetal growth and gestation age could be important not only for research purposes but also for clinical investigations, evaluating if foetal or adnexal anomalies occur [4, 10-12] and when the date of mating is unknown, to provide useful information for breeding purposes [6, 13, 14].

Screening gestational progression by ultrasonography could also be important in animals that are inserted in assisted reproduction programs and are recipients of IVP embryos. In fact alteration of foetal development have been reported after transfer of IVP embryos, cryopreservation and embryo cloning, mainly due to alteration of gene expression during the early

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cleavage stages [15, 16]. Production of embryos in vitro and their following cryopreservation exposes them to hazards not normally encountered in vivo and could result to unforeseen consequences including an increase of abnormal growth and foetal development called "large offspring syndrome" (LOS) [17]. LOS is characterized by a multitude of pathologic changes, of which extended gestation length and increased birth weight are predominant features [2, 17]. Severity of these pathologies can be different according to the in vitro embryo production procedures. For these reasons researchers addressed their efforts to reduce negative consequences of in vitro culture by the definition of specific systems able to overcome or reduce the relatives fetuses alterations [2, 18].

The aim of our study was to create a data set of morphometric parameters during foetal growth by using ultrasonography in Sarda natural mated ewes during different stages of gestation and to compare these data with those of IVP/V/W embryos transferred in recipient ewes.

MATERIALS AND METHODS

All chemicals in this study were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless stated otherwise.

Experiment 1: 20 adult Sarda ewes were synchronized using intravaginal 40mg fluorogestone acetate sponges (Cronogest Intervet® S.r.l. Italy) during 14 days and an administration of 333 U.I./ewe of PMSG (Cronogest Intervet® S.r.l. Italy) on the day of the sponge removal. Ewes were left with two fertile rams of the same Sarda breed for 5 days and naturally mated. The day of mating was designed as day 0 of pregnancy. On day 21th after mating pregnancy diagnosis was performed by transabdominal ultrasonography. From day 35th of pregnancy we started to register the three morphometric parameters, CRL, BPD and TL. All ultrasound foetal measurements were performed using a Philips HD 11 and a 5-8 MHz microconvex probe.

Experiment 2:

Embryos Production: Ovaries were collected form adult Sarda ewes at local slaughterhouses and transported to the laboratory in Dulbecco phosphate buffered saline (PBS) with antibiotics. Collection of cumulus–oocyte complexes (COCs) was performed in sterile Petri dishes containing 20mM Hepes-buffered TCM 199 supplemented with 0.1% polyvinyl alcohol and antibiotics. Only COCs showing several intact cumulus cell layers and uniform cytoplasm with homogenously distributed lipid droplets were selected and matured in vitro in TCM 199 supplemented with 10% heat-treated oestrus sheep serum (OSS), 0.1 IumL-1 FSH, 0.1 IUmL-1LH and 100 mM cysteamine. COCs were cultured for 24 h in 5% CO_2 in air at 38.5°C in four-well Petri dishes with 600 mL of maturation medium, layered with 300 mL of mineral oil.

In vitro-matured oocytes were fertilized in SOF medium +2% OSS for 22 h at 38.58C and 5% CO_2, 5% O_2 and 90% N_2 under mineral oil in four-well Petri dishes with Sarda ram frozen–thawed spermatozoa selected by swim-up technique ($1x10^6$ spermatozoa mL^{-1}).

IVF zygotes were cultured for 7 days in groups in four-well Petri dishes in SOF + essential and non-essential amino acids at oviductal concentration, + 0.4% BSA under mineral oil, in maximum humidified atmosphere with 5% CO_2, 5% O2 and 90% N_2 at 38.5°C.

Vitrification was performed following the Minimum Essential Volume (MEV) method using cryotops as device. Embryos were initially equilibrated at 38.5°C for 1 min in holding medium (HM) consisting of 20 mM Hepes-buffered TCM 199 supplemented with 20% (v/v) fetal calf serum (FCS). After equilibration, the embryos were incubated in 7.5% (v/v) ethylene glycol (EG) + 7.5% (v/v) dimethylsulfoxide (DMSO) in HM for 3 min and then transferred to 16.5% (v/v) EG and 16.5% (v/v) DMSO and 0.5 M sucrose in HM for 20 s. The embryos were then loaded on cryotops and then immediately plunged into liquid nitrogen (LN_2) for storage for at least one week. For warming, cryotops were directly inserted in HM supplemented with 1.25 M sucrose for 1 min. Embryos were then transferred into HM at decreasing sucrose concentrations (0.62 and 0.31 M) for 30 seconds, then washed in HM. Warmed embryos were thereafter cultured for 3h in TCM 199 supplemented with 10% heat-treated oestrus sheep serum and then transferred into a recipient ewe.

Embryo-Transfer: 30 recipient ewes were pre-synchronized with the same progestagen treatment (Cronogest Intervet® S.r.l. Italy) as the natural mating group. The transfers were performed by using a laparotomic technique on day 7 after the onset of the estrus. After an incision on the abdominal wall about 5 cm below of the umbilical scar, the reproductive tract was exteriorized to assess the corpus luteum (CL). With the use of a catheter, 3 vitrified/warmed blastocysts per recipient sheep were transferred into the top of the uterine horn.

As in the first group, pregnancy diagnosis were performed on day 21th (14 days after transfer) and from day 35th of pregnancy (28 days after transfer) we started to register the three morphometric parameters, CRL, BPD and TL. All ultrasonographic foetal measurements were performed using a Philips HD 11 and a 5-8 MHz microconvex probe.

Statistical Analysis: Data were analyzed by MINITAB Release 12.1. Significance level was set at $P \leq 0.05$.

RESULTS

Experiment 1: We were able to achieve a pregnancy diagnosis on day 21th by transabdominal ultrasonography. 19 ewes out of 20 were effectively pregnant (95%) and in 3 cases we noticed the presence of 2 embryos. However only single gestations were used to determine the morphometric parameters by ultrasonography. All ewes gave birth between 148th and 151th day of pregnancy and expulsion of placenta occurred spontaneously. Newborns weighed between 2.8 and 3.6 kg. CRL growth was rapid and constant proceeding from 1.17 cm on 30th day to 2.65 cm on 40th day. For this reason, it is not possible to measure the conceptus in its total length after 40th day and afterwards it is more advisable to evaluate other parameters. Similarly BPD growth trend showed a regular evolution in examined period, proceeding from 1.4 cm on 50th day to 3.66 cm on 100th day; at a later stage is very difficult to examine this parameter, due to the increase of foetal movements and reduced foetus-membranes area. TL showed an increasing regular trend from 54th until 89th day, when it starts a faster raise until 100 th day (3.12 cm on day 89th, 5.24 cm on day 100 th). This occurrence is probably due to the ossification of the epiphysis and for this reason ultrasound measurement appears increased. Tables 1, 2 and 3 show registered data of N/M group.

Experiment 2: 14 days after embryo transfer we detected pregnancy in 20/30 foster ewes (66%). All ewes had offspring without macroscopical defects or severe abnormalities. However in 7 cases out of 20 (35%) we detected the absence of spontaneous delivery and a caesarian section on day 153th was necessary. The presence of diffuse calcifications in the placenta's vessels were observed in almost all recipients of vitrified/warmed blastocysts. Comparing the morphometric parameters during the foetal growth no significant differences were observed between the NM and V/W group in the studied period. CRL trend, as described in Graphic 1, was comparable in the two groups examined (1.17 cm on day 30th – 2.45 cm on day 40th for NM group and 1.18 cm on day 30th – 2.52 cm on day 40th for V/W group). Graphic 2 shows how BPD trend is quite different in the two groups with significant differences ($P \leq 0.05$) on day 62th (2.4 cm for NM ewes and 1.87 cm for V/W ewes) and on day 72th (2.55 cm for NM ewes and 2.94 cm for V/W ewes). The growth trend is more regular and constant in V/W group, while in NM group we noticed a slower increase from day 65th to day 80th to reach at day 100th the same value of V/W group. TL parameters increased faster from day 89th to day 100th in the two groups: this rise is probably due to the ossification of the bone as described in Graphic 3. Tables 1, 2 and 3 show registered data of V/W group.

Table 1: Mean of CRL data registered on NM and V/W ewes in cm

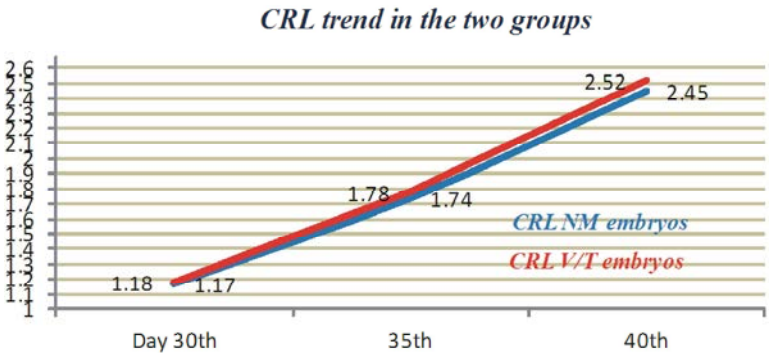
	CRL in NM and V/W ewes	
	NM	V/W
Day 30th of pregnancy	1.17 ± 0.04	1.18 ± 0.0513
Day 35th of pregnancy	1.74 ± 0.26	1.78 ± 0.342
Day 405th of pregnancy	2.45 ± 0.35	2.52 ± 0.343

Table 2: Mean of BPD data registered on NM and V/W ewes in cm

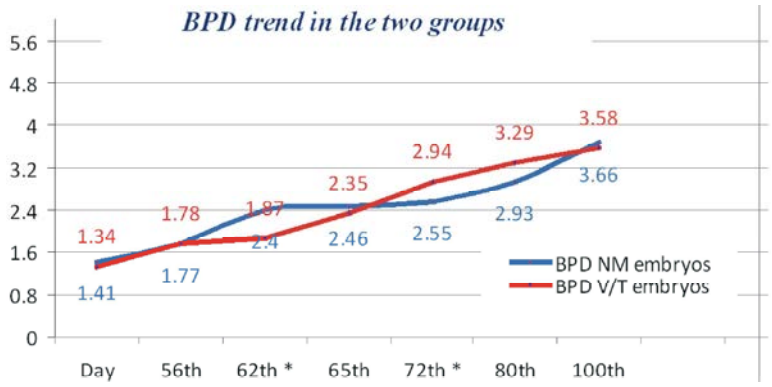
	BPD in NM and V/W ewes	
	NM	V/W
Day 50th of pregnancy	1.41 ± 0.08	1.34 ± 0.08
Day 56th of pregnancy	1.77 ± 0.18	1.78 ± 0.10
Day 62th of pregnancy	2.40 ± 0.02	1.87 ± 0.28
Day 65th of pregnancy	2.46 ± 0.13	2.35 ± 0.07
Day 72th of pregnancy	2.55 ± 0.06	2.94 ± 0.34
Day 80th of pregnancy	2.93 ± 0.15	3.29 ± 0.06
Day 100th of pregnancy	3.66 ± 0.07	3.58 ± 0.04

Table 3: Mean of TL data registered on NM and V/W ewes in cm

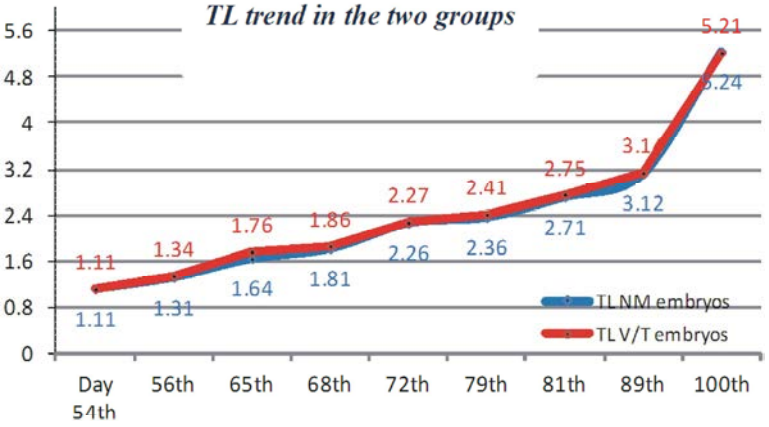
	TL in NM and V/W ewes	
	NM	V/W
Day 54th of pregnancy	1.11 ± 0.17	1.11 ± 0.21
Day 56th of pregnancy	1.31 ± 0.15	1.34 ± 0.17
Day 65th of pregnancy	1.64 ± 0.13	1.76 ± 0.17
Day 68th of pregnancy	1.81 ± 0.07	1.86 ± 0.07
Day 72th of pregnancy	2.26 ± 0.23	2.27 ± 0.28
Day 79th of pregnancy	2.36 ± 0.09	2.41 ± 0.17
Day 81th of pregnancy	2.71 ± 0.01	2.75 ± 0.01
Day 89th of pregnancy	3.12 ± 0.29	3.14 ± 0.33
Day 100th of pregnancy	5.24 ± 0.31	5.21 ± 0.42



Graphic 1: Trend of CRL in the two groups examined. Significance level P ≤ 0.05



Graphic 2: Trend of BPD in the two groups examined. Significance level P ≤ 0.05



Graphic 3: Trend of TL in the two groups examined. Significance level P ≤ 0.05

DISCUSSION

Our aim was to provide a chronological description of the development of the ovine conceptus between 30th and 100th day, based on 3 different ultrasonographic morphometric foetal parameters: CRL, BPD and TL and to compare these data with ones registered during foetal growth of V/W/ IVP embryos transferred into recipients ewes. Our findings showed a regular and costant growth of the CRL and BPD whereas TL showed a rapid increase from 89th to 100th day. CRL and BPD could better represent the age of foetus when the date of mating is unknown. Not many significant differences of these three morphometric parameters were observed between the NM and V/W embryos group, but as previously reported we observed an increase of the gestation period in the V/W group and the absence of spontaneus lambing. Newborns derived after transfer of IVP/V/W embryos did not show any macroscopical alterations as well as the control group.

From 1980's up to now several parameters have been used to monitor foetal growth and foetal well being in human [8], equine [19, 20] and ovine [6, 7] by various authors. In Robinson *et al.* [5] produced a mathematical model for the description of foetal growth from 55th to 145th days of gestation in ewes. Values obtained in early pregnancy are more accurate for the estimation of the gestational age compared to those obtained in later stages that are affected by the individual genetic background too [7]. CRL has been frequently used in postmortem foetal observations in different species and it is considered one of the most representative measures [3]. Concerning ruminants similar morphometric parameters have been considered. In bovine the most reliable foetal anatomic parameters for estimation of gestational age are: biparietal diameter of the skull [21], the head diameter [14], the uterine diameter and CRL. Previous studies with a 5 MHz external probe showed that the BPD is the most representative parameter of the gestational age during the second third of pregnancy in sheep [22]. However, it is not possible to obtain symmetric images of the skull before 40–50 days of pregnancy [23]. Aiumlamai *et al.* [9] created a grid monitoring seven Swedish Pelt sheep ewes and correlated by means of a simple regression analysis: heart rate, biparietal diameter of the skull and diameter of the body trunk with gestational age. They showed that BPD parameter was a measurement as good as the diameter of the body trunk for estimating the age of the fetuses. Barbera *et al.* [8] used 43 mixed Rambouillet-Columbia ewes with a singleton pregnancy to monitor BPD and other morphometric parameters and compared the obtained data with analogous of human foetuses. They evidenced that during the gestation BPD, TL and femur length in human foetus show a deceleration of growth, whereas in the sheep deceleration is evident only for BPD. Femoral and Tibia lengths show an acceleration of the growth rate, with TL growing faster than FL. For this reason the ovine tibia is a more sensitive index of foetal growth than the femur, which is a standard bone measurement in humans [8].

In our study we noticed only two significant differences between the morphometric parameters of the NM ewes and those of IVP/V/W embryos in the examined period for BPD measurement. Furthermore an increase of the gestation period in the V/W group was observed. In 7 recipient ewes we detected that were no birth signal until 153th day of pregnancy and we had to make a caesarean section. Previous studies confirmed that cryopreservation extends the gestation period by at least four days, probably due to a slow down of cryopreserved embryo cellular activity [18, 24]. Some authors demonstrated that foetuses derived from the fresh transfer of IVP embryos tend to be heavier and longer than their in vivo counterparts. The reasons for this dissimilarity are unclear. The most striking feature of the syndrome is large size at birth and a lamb of five times birth weight has been reported by Walker *et al.* [18]. Gestation is frequently extended, although this is insufficient to justify the increased birth weight [18]. Other authors affirmed that increases in birth weight apparently occurs independently on the increase in gestation length [25]. Walker *et al.* [18] also described other features apparently associated with the syndrome at birth, including breathing difficulties, reluctance to suckle and sudden perinatal death [18]. Furthermore dystocia associated with increased birth weight often requires delivery by Caesarean section [26]. Increased prenatal losses, particularly in the first half of pregnancy [24], are associated with the syndrome too [18]. As indicated by other authors the delay in parturition and the uterine inertia could be the consequences of placenta defects [11]. These events have been also observed after transfer of embryos derived from nuclear transfer technologies. Many of these abnormalities could be overcame by appropriate culture conditions. The exclusion of serum in the culture media significantly reduces the foetal growth anomalies. Our culture condition and cryopreservation procedures seem also to reduce the weight abnormalities but not the deficiency related to the parturition signals. The problems that occur during the delivery stages could be also due to the diffuse calcifications observed in the placenta that can modify its physiological functions. The role of these alterations in parturition dynamic defaults have to be further investigated. Our study described for the first time by ultrasound morphometric measurements the foetal growth in Sardinia ewes from 30th up to 100th day of gestation. We compared the data with those obtained from V/W/IVP foetuses in order to verify the presence of alterations during the foetal development. Data generated can be used for experimental studies as well as for breeding purpose.

In conclusion we can adfirm that our work shows the feasibility of the ultrasonographic technique for the monitoring of pregnancy during the first 100 days in the two groups of ewes. At the end of the studied period no significant differences were observed between the morphometric parameters of NM (natural mating) group and V/W (Vitrified/Warmed embryos) group, even if was observed an in increase of the gestation period in the vitrified/warmed group and the absence of spontaneous

lambing in 35%. Newborns derived from V/W group did not show any macroscopical alterations as well as the control group. Future investigations into metabolic embrional alterations between the two studied groups are desirable and important to discover how much vitrification process affect the embryo during gestation.

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