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PREGNANCY DIAGNOSIS IN SHEEP

Doctoral thesis

OF

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Budapest
2003

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……
Aly Mohamed Aly Karen

Dedication

To the spirit of my father
And to my mother,
my wife,
and my kids, Omar and Abdel Rahman

CONTENTS

LIST OF ABBREVIATIONS

bpm .....beat per minute
BSA.....bovine serum albumin
boPAG.....bovine pregnancy-associated glycoprotein
caPAG .....caprine pregnancy-associated glycoprotein
cpm.....counts per minute
eCG .....equine chorionic gonadotrophin
^{125}I .....ioden 125
IgG .....immunoglobulin G
i.m. .....intramuscular(ly)
IU .....international unit
KDa .....kilo dalton
M .....molar
mg .....milligram
MHz .....mega hertz
ng/mL ..... nanogram per milliliter
ovPAG.....ovine pregnancy-associated glycoprotein
P .....probability
P4 .....progesterone
PAG-RIA .....pregnancy associated glycoprotein-radioimmunoasssay
PSPB .....pregnancy-specific protein B
PEG.....polyethelen glycol
RIA.....radioimmunoassay
SD .....standard deviation

INTRODUCTION

The intensive sheep management and the wide spread application of the controlled breeding techniques, such as artificial insemination and out-of season breeding, increase the need for an accurate and practical test for early pregnancy diagnosis. The traditional methods such as non-return to estrus and abdominal ballotment are not satisfactory. In addition, laparotomy, laparoscopy, rosette inhibition test and vaginal biopsy are accurate techniques, however these methods are impractical under farm conditions (Goel and Agrawal, 1992; Gordon 1999). Methods of pregnancy diagnosis depending on visualization of the conceptus or determination of its secretory products in the maternal blood or in the milk are the most accurate and specific methods for pregnancy. In 1980, B-mode ultrasonography was introduced in the veterinary field and used for pregnancy diagnosis in mare (Palmer and Driancourt, 1980) and then received large acceptance for diagnosing pregnancy in all domestic animals (Kähn, 1992). Transrectal ultrasonography has been recommended as a simple, rapid and practical method for early pregnancy diagnosis in sheep (Buckrell et al., 1986). However, the accuracy of this technique is greatly variable (Gearhart et al., 1988, Garcia et al., 1993; Kaufluss et al., 1996). Recently, pregnancy-associated glycoproteins (PAG) have been isolated from domestic ruminant placentas (Zoli et al., 1991 and 1995; Garbayo et al., 1998) and radioimmunoassays have been developed for their determination in the maternal plasma (Zoli et al., 1992, Rannilla et al., 1994, Perényi et al., 2002) or in the milk (González et al., 2000). In cattle and goats, the pregnancy-associated glycoprotein radioimmunoassays (PAG-RIA) accurately diagnose early pregnancy (Szenci et al., 1998; González et al., 1999). However, there is no data concerning the accuracy of PAG-RIA test for early pregnancy diagnosis in sheep.

The reliability of the diagnostic method and the accuracy of the diagnosis can be evaluated using a 2 x 2 table for which data have to be obtained for all four cells (Smith, 1991, Table 1).

Two parameters are traditionally used for describing the accuracy of the diagnostic methods. The sensitivity (Se) is defined as the likelihood of a positive test result in ewes

known to be lambed. It is calculated by the following equation; Se = 100 x a/(a+d). Conversely, the specificity (Sp) is defined as likelihood of a negative test result in ewes known to be of non-pregnant and it is calculated by the following equation; Sp = 100 x c/(c+b). Besides the above-mentioned parameters, the practitioner should be concerned with the predictive value of the diagnostic test i.e. the probability that the diagnosis reflects the true pregnancy status. The positive predictive value (+PV) would then be the probability of the presence of pregnancy in an animal diagnosed pregnant i.e. 100 x a/(a+b). The negative predictive value (-PV) would be the probability of absence of pregnancy in an animal diagnosed non-pregnant i.e. 100 x c/(c+d) (Hanzen et al., 2000).

Table 1

Outcome of diagnostic tests

Diagnosis	**Positive**	**Negative**
Positive	a (correct positive)	b (incorrect positive)
Negative	d (incorrect negative)	c (correct negative)

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THE PURPOSE OF THE THESIS

This work was undertaken to find the most accurate method for early pregnancy diagnosis in Awassi x Merino ewes. For this purpose:

A) the accuracy of the PAG–RIA test for pregnancy diagnosis was evaluated and compared with that of progesterone test.

B) the factors which may affect the accuracy of transrectal ultrasonography were investigated.

And C) the false transrectal ultrasonogragraphic pregnancy diagnoses were evaluated by measuring plasma level of ovPAG.

CHAPTER 1

PREGNANCY DIAGNOSIS IN SHEEP: REVIEW OF THE MOST PRACTICAL METHODS

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Acta Veterinaria Brno

2001, 70:115-126

ABSTRACT

Various practical methods have been used for pregnancy diagnosis in sheep. Both pregnancy and fetal numbers are accurately diagnosed by using radiography after Day 70 of the gestation. Rectal-abdominal palpation technique detects pregnancy with an accuracy of 66 to 100% from Days 49 to 109 of gestation, however it has a low (17 to 57%) accuracy for determining multiple fetuses. Progesterone assays have a high sensitivity (88% to 100%) and a low specificity (60% to 72%) at Days 16 to 18. Estrone sulphate assay accurately detects pregnant ewe at Days 30 to 35. Ovine pregnancy specific protein B (ovPSPB) assay accurately (100%) detects pregnancy from Days 26 after breeding onwards. The accuracy of progesterone, estrone sulphate and ovPSPB assays for determining fetal numbers is relatively low. A-mode and Doppler ultrasonic techniques accurately detect pregnancy during the second half of gestation. Fetal numbers can not be determined by A-mode ultrasound, while the Doppler technique needs experience to achieve high accuracy. Transrectal B-mode, real time ultrasonography identifies the embryonic vesicles as early as Day 12 after mating, but the sensitivity of the technique for pregnancy is very low (12 %) earlier than 25 days after mating. Transabdominal B-mode ultrasonography achieved high accuracy for pregnancy diagnosis (94 % to 100 %) and the determination of fetal numbers (92 % to 99 %) at Days 29 to 106 of gestation. Real-time, B-mode ultrasonography appears to be the most practical and accurate method for diagnosing pregnancy and determining fetal number in sheep.

Keywords: pregnancy diagnosis; ewe; radiography; rectoabdominal palpation; hormonal assays; pregnancy proteins; ultrasonography

INTRODUCTION

Early detection of pregnancy is of considerable economic value to sheep industry. Non pregnant ewes could be sold, reducing feed expenses, while non-pregnant lambs could be marketed at higher price than they would bring as mature ewes (Gearhart et al., 1988).

Separation of the sheep flocks into pregnant and non-pregnant ewes might reduce reproductive and production losses in form of abortions, stillbirths and production of weak lambs (Wani et al., 1998).

Predictions of the number of fetuses would allow appropriate nutritional management of the ewes in late gestation that will prevent pregnancy toxemia (Ford, 1983), minimize prelambing feeding costs, optimize birth weight, weaning weight and survivability of lambs and reduce the incidence of dystocia (Gearhart et al., 1988). In addition, the accurate information on the stage of gestation would be useful to dry off lactating females at adequate period and to monitor the females near term (Doize et al., 1997).

METHODS OF PREGNANCY DIAGNOSIS

Various methods have been used to diagnose pregnancy in sheep. These methods can be classified as less practical such as the management method (non-return to estrus), abdominal palpation and ballotment, palpation of the caudal uterine artery, laparotomy, peritoneoscopy and rossete inhibition test reviewed by Ishwar (1995), and the most practical methods such as radiography, rectal abdominal palpation, hormonal assays, pregnancy protein assays and ultrasonography. In the present review, only the most practical methods are discussed.

1. RADIOGRAPHY

Ford et al. (1963) examined 322 ewes by radiography and reported 100 % and 90 % accuracy for diagnosing pregnancy and determination of the fetal number, respectively after 70 days of gestation. Grace et al. (1989) reported 94 to 100% accuracy of radiography for determining fetal numbers in 13 sheep flocks. Besides the accuracy, the technique is quick; 400 to 600 ewes can be tested per day under farm conditions. The cost of the equipment and the potential health hazard to the operator may limit its use in the field (West, 1986).

2. RECTAL ABDOMINAL PALPATION

Pregnancy diagnosis in sheep was determined by gentle insertion of a lubricated glass rod (1.5 cm in diameter and 50 cm long) into the rectum of ewe lying on its back. The free hand was placed on the posterior abdomen while the rod was manipulated with the other hand (Hulet, 1972). At the early stage of pregnancy, the sensitivity of the technique for

diagnosing pregnancy was low but it increased with progressing of the pregnancy reaching the highest accuracy (100 %) at Days 85 to 109 after mating (Hulet 1972; Chauhan et al., 1991; Table 1). In contrast, others (Tyrrell and Plant, 1979; Trapp and Slyter, 1983) reported a lower sensitivity and specificity at Days 60 to 96 after mating (Table 1). Although this technique is simple, cheap and quick (150 ewes can be examined per hour), it had a low accuracy in diagnosing multiple fetuses (Table 2) and was more hazardous with respect to rectal injury (Tyrrell and Plant, 1979) and abortion (Turner and Hindson, 1975; Ishwar, 1995).

Table 1. Sensitivity (Se), specificity (Sp), and predictive (+PV, -PV) values of rectal abdominal technique for pregnancy diagnosis in sheep

No. of animals	Days of exam.	a	b	c	d	Se %	Sp %	+PV %	-PV %	Authors
79	85 to 109	61	0	18	0	100	100	100	100	Hulet, 1972
432	21 to 55					59				Tyrrell & Plant 1979
99	49 to 83					73				Tyrrell & Plant 1979
498	60 to 96	173	97	139	89	66	59	62	61	Trapp & Slyter 1983
14		10	2	2	0	100	50	82	100	Chauhan et al.,1991

a, correct positive (pregnant); b, false positive (non pregnant); c, correct negative (non pregnant); d, incorrect negative (pregnant).

Table 2. Sensitivity (Se), specificity (Sp), and predictive (+PV, -PV) values of rectal abdominal technique in determination of fetal numbers

No. of animals	Days of exam.	a	b	c	D	Se %	Sp %	+PV %	-PV %	Authors
41	90 to 105	4	1	33	3	57	97	80	92	Hulet (1973)
12		1	1	5	5	17	83	50	50	Chauhan et al. (1991)

a, correct positive (multiple); b, false positive (single); c, correct negative (single); d, false negative (multiple).

The technique of bimanual palpation of small ruminants was developed by Kutty and Sudarsanan (1996). This method includes digital palpation per rectum combined with abdominal manipulation. By using this technique pregnant ewes (n = 9) were accurately diagnosed based on enlarged cervix, prepubic position of the uterus, palpation of

placentomes and /or fetal parts, asymmetry and /or marked distension of uterine horns and inability to palpate the ovaries (Kutty, 1999).

3. HORMONAL ASSAYS

3.1. Assessment of progesterone

Measurement of blood progesterone concentration is a reliable indicator of the functional *corpus luteum*. Concentration of plasma progesterone samples was determined in ewes at Day 18 post-breeding by using enzyme immunoassay (EIA) and radioimmunoassay (RIA). The accuracy of both type of assays for detecting pregnancy was high, while it was low for diagnosing non-pregnancy (Amezcua-Moreno, 1988; Susmel and Piasentier, 1992; Gvozdic and Ivkov, 1994; Table 3). On the other hand, 100 % accuracy for detecting non pregnant ewes was achieved by using EIA at Day 16 (McPhee and Tiberghien, 1987) and Day 21 after mating (Zarkawi, 1997) or by using RIA at Days 17 to 18 (Zarkawi et al., 1999; Table 3). Early embryonic death, uterine and/ or ovarian pathology may be the source of the false positive cases. At Days 100 ± 9 after breeding, the accuracy of progesterone assay for pregnancy diagnosis was 98% in ewe lambs and 99 % in mature ewes (Schneider and Hallford 1996).

Table 3. Sensitivity (Se), specificity (Sp), and predictive (+PV, -PV) values of progesterone assay for diagnosing pregnancy in sheep

Days of exam.	No. of animals	a	b	c	d	Se %	Sp %	+PV %	-PV %	Authors
16 to 17	130	106	0	24	0	100	100	100	100	McPhee & Tiberghien (1987)
18	170					91	64			Amezcua-Moreno (1988)
18	112	80	9	23	0	100	72	90	100	Susmel & Piasentier (1992)
16 to 18	22	15	2	3	2	88	60	88	60	Gvozdic & Ivkov (1994)
21	16	16	0	0	0	100		100		Zarkawi (1997)
17 to 18	24	24	0	0	0	100		100		Zarkawi et al. (1999)

a, correct positive (pregnant); b, false positive (non pregnant); c, correct negative (non pregnant); d, false negative (pregnant).

Enzyme immunoassay (EIA) test for the measurement of fecal immunoreactive Pregnendiol–3-Glucuronide (iPdG), a progesterone metabolite, was a useful tool for diagnosing pregnancy in Big horn sheep with 100 % accuracy from about Day 60 of pregnancy until a few days before parturition. (Borjesson et al., 1996).

Concerning the estimation of the fetal number, serum progesterone concentration was significantly higher in ewes carrying two and three fetuses than those carrying one fetus (19.2 and 29.9 ng/ml, vs 9.2 ng/ml, respectively) (Chauhan and Waziri,1991). There was a positive relationship between the number of fetuses and the mean plasma progesterone concentrations ($P<0.001$) after the second half of pregnancy (Kalkan et al.,1996). The number of fetuses was estimated with 88% accuracy in ewe lambs and with 74% accuracy in mature ewes at Days 100 ± 9 after breeding (Schneider and Hallford, 1996). In contrast, others reported a much lower accuracy (25%) for ewes carrying multiple fetuses (Chauhan et al., 1991; Sandabe et al., 1994).

Regarding the fetal sex, the plasma progesterone concentrations of ewes giving birth to male and female lambs were not significantly different (Kalkan et al., 1996).

3.2. *Assessment of estrone sulphate*

The presence of a viable feto-placental unit is accompanied by an increase in estrone sulphate concentrations in the peripheral plasma of ewes. Estrone sulphate was detectable around Day 70 of gestation with value ranging between 0.1 to 0.7 ng/ml, then its level increased steadily till 2 days before parturition when an upsurge was seen (15-50 ng/ml) (Tsang, 1978). At Day 85 of gestation, there was a significant difference in the level of estrone sulphate between pregnant and non-pregnant ewes. However, due to considerable variation of the hormone levels between individuals, the accuracy for detection of non-pregnancy was only 44 % whilst for detection of pregnancy was 87.9 % using the cut-off value of 0.1 ng/ml (Worsfold et al., 1986). On the contrary, Illera et al. (2000) reported that the EIA test for the measurements of serum estrone sulphate concentrations gave an optimal accuracy for pregnancy diagnosis between Days 30 to 35 of gestation.

Regarding the fetal number, the concentration of serum estrone sulphate was significantly higher in ewes carrying multiple than those carrying single fetus from Days 80 to 124 of gestation (Illera et al., 2000). However, the determination of estrone sulphate

concentrations in ovine blood might not be reliable for prediction of fetal numbers due to the high variation between individuals (Worsfold et al., 1986).

3.3. Ovine chorionic somatommamotrophin (ovCS) or ovine placental lactogen (ovPL)

Ovine placental lactogen (oPL) was studied and purified by Chan et al. (1978). Radioimmunoassay of ovPL achieved 97% and 100 % accuracy for diagnosing pregnant and non- pregnant ewes at Day 64 of gestation, respectively (Robertson et al., 1980).

4. ASSESSMENT OF PREGNANCY PROTEINS

4.1. Pregnancy specific protein B (PSPB)

Pregnancy specific protein B (PSPB) first detected in the bovine placenta (Butler et al., 1982), is secreted by binucleate cells of fetal trophoectoderm (Eckblad et al.,1985). The physiological role of PSPB during pregnancy might be the maintenance of *corpus luteum* by stimulating prostaglandin E2 production (Vecchio et al., 1995).

Although the RIA test for the measurements of bovine (bo)PSPB accurately detects pregnancy (100%) and non pregnancy (83%) in sheep from Days 26 to 106 of gestation (Table 4), ovine PSPB concentration can not be measured quantitatively because ovine antigen cross-reacts only incompletely with antibodies to boPSPB (Ruder et al., 1988).

Table 4. Sensitivity (Se), specificity (Sp) and predictive (+PV, -PV) values of ovPSPB assay for pregnancy diagnosis in sheep

Days of exam.	No. of animals	a	b	c	d	Se %	Sp %	+PV %	-PV %	Authors
26-96	33	30	2	1	0	100	33	94	100	Ruder et al. (1988)
35-106	180	159	2	19	0	100	90	99	100	Ruder et al. (1988)
Total	213	189	4	20	0	100	83	97	100	

a, correct positive (pregnant); b, false positive (non pregnant); c, correct negative (non pregnant); d, false negative.

Willard et al. (1987) developed a quantitative RIA test for the measurements of ovine pregnancy specific protein B (ovPSPB). Ovine PSPB became detectable at 19.7± 0.1 (Mean ± SE) (Willard et al., 1987; 1995) and 21.7± 0.6 days postmating (Wallace et al., 1997). Then, it increased steadily until Day 30 when it was 10.8 ± 0.4 ng/ml. The

concentration remained stable within a period of 20 days prepartum (Willard et al., 1995). After lambing, the concentration dropped rapidly and it was last detectable at 12.8 ± 2.3 days (Willard et al., 1995) and 3 ± 0.1 weeks postpartum (Willard et al., 1987).

By using the RIA test for the measurements of ovPSPB, the accuracy for detecting ewes carrying single and twin lambs was 71% and 81%, respectively from Days 60 to 120 of gestation (Willard et al., 1995). At the same time, ovPSPB concentrations were not influenced by the sex of the fetus (Wallace et al., 1997).

Ovine PSPB might be a useful marker of placental development and function and provide a reliable indicator of fetal distress and adverse pregnancy outcome. Between Days 50 and 100 of gestation, ovPSPB concentrations were positively correlated with placental weight at term. In addition, the mass of the fetus in ewes that aborted during late pregnancy was highly correlated with ovPSPB concentrations up to Day 120 of gestation (Wallace et al., 1997).

4.2. Ovine pregnancy-associated glycoprotein (ovPAGs)

Ovine pregnancy-associated glycoproteins (ovPAGs) are synthesized by binucleate cells of trophoblast, and belong to aspartic proteinase family (Xie et al., 1991) and most of them are without enzyme activity (Xie et al., 1997). They have molecular weights between 43 to 67 kDa (Zoli et al., 1995, Xie et al., 1997).

The concentration of ovPAG in Churra and Merino ewes was detectable in some (20/30) ewes at Week 3 and in all ewes at Week 4 after mating (Ranilla et al., 1994). The concentration of ovPAG increased slowly from Weeks 3 to 9 of gestation. Thereafter, plasmatic profiles of ovPAG varied among sheep breeds from Week 9 till Week17, however, ovPAG concentrations increased in all studied breeds from Week 17 till lambing. After lambing, the ovPAG levels decreased rapidly reaching the basal value at fourth week postpartum (Ranilla et al., 1994 and 1997; Gajewski et al., 1999).

The concentration of ovPAG might be influenced by the fetal numbers and the sex of the fetus. Ewes carrying two fetuses had higher mean ovPAG concentrations than those carrying a single fetus from Week 12 of gestation to lambing. This difference was only significant at Week 21 (Ranilla et al., 1997). Also, ewes carrying male fetuses had ovPAG concentrations higher than those carrying female fetuses at Weeks 19, 20 and 21 of gestation (Ranilla et al., 1994).

Although boPAG1 and caprine (ca) PAG have been successfully used for detecting pregnancy in cattle (Zoli et al., 1992; Szenci et al., 1998) and goats (Folch et al., 1993, Gonzalez et al., 1999), there is no data evaluating the accuracy of ovPAG assays for diagnosing pregnancy in sheep.

5. ULTRASONOGRAPHY

In the past 20 years, three types of ultrasonographic systems were used for pregnancy diagnosis in the small ruminants.

5.1. A-mode ultrasound (Amplitude-depth or echo-pulse)

In this system, the transducer containing one crystal emits ultrasound waves which penetrate the tissues under the skin and are reflected when meet a high acoustic impedance interfaces (pregnant uterus or fluid-filled structures). The transducer receives the reflected echoes and converts it into peaks on oscilliscope with horizontal scale representing the depth of the reflecting structure or into audible signal.

Meredith and Madani (1980) used the reflection of ultrasound at depth 9 cm or greater as a positive sign of pregnancy in ewe and reported 96 % sensitivity and 87.5 % specificity in the period from 61 to 151 days after mating. However, by the same approach, lower sensitivity (86.7 %) and specificity (69 %) was reported in the ewe lambs at Days 73 to 103 postmating (Madel, 1983). By using echo-pulse detectors, the accuracy for detecting pregnant ewes averaged 91% from Days 69 to 112 of gestation (Trapp and Slyter, 1983). However, Watt et al. (1984) reported 97 % accuracy for diagnosing pregnancy from Day 51 of gestation to lambing. A-mode ultrasound is a quick, convenient and simple technique, but it can not predict the fetal number and the viability of the fetus.

5.2. Doppler ultrasound

Doppler devices utilize the Doppler shift principle to detect the fetal heartbeats and flow of blood in uterine and fetal vessels. Lindahl (1971) reported that the intrarectal Doppler technique could be used for diagnosing pregnancy at the beginning of the second third with an accuracy of 90 % or better. According to the work reported by Deas (1977) the accuracy of intrarectal Doppler transducer for diagnosing pregnancy and non-pregnancy was 82 % and 91 %, respectively from Days 41 to 60 of gestation. After Day 71, the accuracy for diagnosing pregnancy and non pregnancy ranged between 85 % and 94 %,

respectively (Watt et al., 1984). In contrast, Trapp and Slyter (1983) reported 68 % and 84 % accuracy for diagnosing pregnancy and non-pregnancy from Days 60 to 96 of gestation. The use of an external Doppler transducer gave almost 100 % accuracy for diagnosing pregnancy after Day 111 of gestation (Watt et al., 1984).

Concerning the predictions of fetal numbers, the external Doppler technique, when used by skilled operator gave 83 % and 93 % accuracy for diagnosing single and multiple fetuses at Days 80 to 95 of gestation, respectively (Fukui et al., 1986). However, Fukui et al. (1984) reported 74% and 89% accuracy for ewes carrying singles and multiples, respectively from Days 60 to 120 of gestation. Doppler devices have not been used successfully for estimating ovine gestational age (Russel and Goddard, 1995).

5.3. Real-time, B-mode ultrasonography

Real-time B-mode ultrasonic scanning of the uterus in sheep appears to offer an accurate, rapid, safe and practical means for diagnosing pregnancy, determination of fetal numbers and estimation of gestational age.

5.3.1. Diagnosis of pregnancy

By using transrectal ultrasonography (7.5 MHz), embryonic vesicle of the pregnant Manchega dairy ewe was identified at Day 12 after mating, while the first visualization of the embryo was at Day 19 (Gonzalez et al., 1998) or Day 20 (Schrick and Inskeep, 1993). By using 5 MHz transrectal probe, the first signs of pregnancy in form of circular and elongated anaechoic images located in utero cranial to bladder were observed in ewe at Days 17 to 19 (Garcia et al., 1993; Doize et al., 1997), while embryo could be detected at Day 25 after mating (Buckrell et al., 1986).

The specificity of 7.5 MHz transrectal ultrasonography for diagnosing non-pregnancy was low during the first two months of gestation (Schrick and Inskeep, 1993; Table5). The false positive diagnoses were attributed to embryonic or fetal death. The sensitivity of 5 MHz transrectal ultrasonography for detecting pregnant ewes was greatly variable (12 % to 98.7 %) at less than Day 25 of gestation (Gearhart et al., 1988). Thereafter, the sensitivity increased with progressing the pregnancy and ranged between 65 % and 87 % at Days 25 to 50, depending on the breed, age and parity of the ewes, experience of the operator and the technique of the examination (Buckrell et al., 1986; Gearhart et al., 1988; Garcia et al., 1993; Table 5).

Table 5. Sensitivity (Se), specificity (Sp) and predictive (+PV, - PV) values of using transrectal (5 MHz and 7.5 MHz) ultrasonography for pregnancy diagnosis in sheep.

Day of exam.	MHz	No. of animal	a	b	c	d	Se %	Sp %	+PV %	-PV %	Authors
25 to 50	5	64	33	1	25	5	87	96	97	83	Buckrell et al. (1986)
0 to 25	5	26					12	100			Gearthart et al. (1988)
26 to 50	5	26					65	100			Gearthart et al. (1988)
24 to 26	5	91	17	3	62	9	65	95	85	87	Garcia et al. (1993)
32 to 34	5	91	22	1	64	4	85	98	96	94	Garcia et al. (1993)
0 to 60	7.5	117	94	8	13	2	98	62	92	87	Schrick & Inskeep (1993)

a, correct positive (pregnant); b, false positive (non pregnant); c, correct negative (non pregnant); d, false negative (pregnant).

Table 6. Sensitivity (Se), specificity (Sp) and predictive (+PV, - PV) values of using transabdominal (3, 3.5 and 5 MHz) ultrasonography for pregnancy diagnosis in sheep

Days of exam.	MHz	No. of animals	a	b	c	d	Se %	Sp %	+PV %	-PV %	Authors
46 to 106	3.5	5530	5006	1	491	32	99	100	100	94	Fowler & Wilkins (1984)
46 to 93	3.5	554	520	0	34	0	100	100	100	100	White et al. (1984)
29 to 89	3	724	593	3	123	5	99.2	97.6	99.4	96.1	Taverne et al. (1985)
50 to 100	3.5	516	473	0	37	6	99	100	100	88	Davey (1986)
< 40 to > 100		2499	2331	21	141	6	100	87	99	96	Logue et al. (1987)
51 to 75	5	26	24	0	2	0	100	100	100	100	Gearhart et al. (1988)

a, correct positive (pregnant); b, false positive (non pregnant); c, correct negative (non - pregnant); d, false negative (pregnant)

By using transabdominal approach, pregnancy was first verified at Day 25 (Gearhart et al., 1988) or Day 30 after breeding (Bretzlaff et al., 1993). The sensitivity and specificity of the technique were high after Day 29 (Taverne et al., 1985) reaching approximately 100% from Days 46 to 106 of gestation (White et al., 1984; Fowler and Wilkins 1984;

Davey 1986; Gearhart et al., 1988). However, Logue et al. (1987) reported a lower specificity at Days less than 40 to 100 after mating (Table 6).

5.3.2. Determination of the fetal number

By using transrectal ultrasonography (7.5 MHz), single and multiple pregnancies in sheep were accurately (15 of 17 ewes) detected at Day 25 (Schrick and Inskeep, 1993). However, the accuracy of a 5 MHz transrectal ultrasonography for detecting ewes carrying two fetuses or more was disappointing (Gearhart et al., 1988; Table 7). By using transabdominal ultrasonography, the accuracy of experienced operator for determination both single-and multiple-bearing ewes was 99 % from Days 46 to 93 of gestation (White et al., 1984). A similar accuracy for ewes carrying single fetus was reported by Fowler and Wilkins (1984), Davey (1986) and Gearhart et al. (1988), however, a lesser accuracy for ewes carrying multiples was reported by others (Table 7).

Table 7. Sensitivity (Se), Specificity (Sp) and Predictive (+PV, -P V) values of using transrectal (TR) and transabdominal (TA) ultrasonography for determination of fetal numbers in sheep

Days of exam.	Method of exam.	No. of animals	a	b	c	d	Se %	Sp %	+PV %	-PV %	Authors
46 to 106	TA	5039	1328		3577		94	99	99	98	Fowler &Wilkins (1984)
46 to 93	TA	520	327	1	190	2	99	99	100	99	White et al. (1984)
45 to 77	TA	210	142	5	53	10	93.4	91.3	96.5	84.1	Taverne et al. (1985)
50 to 100	TA	479	118	0	349	12	91	100	100	97	Davey (1986)
<40 to >100	TA	2348	1216		1006		96	94	95	94	Logue et al. (1987)
26 to 50	TR	24					5	80			Gearhart et al. (1988)
51 to 75	TA	24					97	100			Gearhart et al. (1988)

a, correct positive (multiple); b, false positive (single); c, correct negative (single); d, false negative (multiple).

5.3.3. Estimation of gestational age

When the date of mating is unknown, monitoring fetal development allows estimation of

gestational age.

A. Embryonic vesicle

Gonzalez et al. (1998) measured the ovine embryonic vesicle from Days 12 to 29 of gestation by using 7.5 MHz transrectal ultrasonography and found a close correlation (r = 0.76) with the gestational age.

B. Crown-Rump length

By using transrectal ultrasonography (7.5 MHz), Schrick and Inskeep (1993) measured the crown-rump length of the ovine fetus from Days 20 to 40 of gestation and described the relationship between the crown-rump length (x) and the gestational age (y) by the following equation, $Y=14.05 +1.16x - 0.012x^2$. By using the same approach, Gonzalez et al. (1998) reported a high (r = 0.94) correlation between the crown- rump length and the gestational age from Days 19 to 48 of gestation.

C. Fetal head diameters

Fetal head diameters including the biparietal diameter, the occipito-nasal length and the diameter of the orbit were used to predict the stage of gestation in sheep.

Regarding to the biparietal diameter (BPD), Gonzalez et al. (1998) used the transrectal ultrasonography to measure the BPD of Manchega sheep from Days 32 to 90 and found a high correlation (r = 0.96) between the measured diameters and the gestational age. Similar correlation was found by using transabdominal approach in Suffolk and Finn sheep from Days 40 to 95 (Haibel and Perkins, 1989), in Booroola x South Australian Merino sheep from Days 49 to 109 (Sergeev et al., 1990) and in Swedish peltsheep from 10 weeks before lambing to birth (Aiumlamai et al., 1992).

Kelly and Newnham (1989) found the occipito-nasal length to be more accurate than BPD, showing a linear increase till Day 80. However, Sergeev et al. (1990) reported that the occipito-nasal length was more difficult to be measured than BPD and had the same accuracy for predicting fetal age. Gonzalez et al. (1998) found a high correlation (r = 0.95) between the fetal occipito-nasal length and the gestational age from Days 38 to 91 of gestation.

Regarding the diameter of the fetal orbit, Gonzalez et al. (1998) reported that the ovine fetal orbit increased in diameter from 2 mm at Day 36 to 17 mm at Day 90 of gestation and it gave a high correlation (r = 0.92) with the fetal age.

D. Thoracic diameter

Ultrasonographic measurements of the ovine fetal thoracic diameter showed high correlation with the fetal age from Days 49 to 109 (Sergeev et al., 1990) and from Days 23 to 90 of gestation (Gonzalez et al., 1998).

E. Fetal heart rate

By using 7.5 MHz transrectal ultrasonography, the rhythmic pulsations within the ovine embryonic vesicle were first detected at Day18 or 19 after mating (Schrick and Inskeep, 1993), while by using 5 MHz transrectal ultrasonography, they were first observed from Days 21-23 after mating (Garcia et al., 1993). Aiumlamai et al. (1992) measured the ovine fetal heart rate during the second half of pregnancy by using transabdominal ultrasonography and reported that the fetal heart rate reached the plateau at 7 weeks before lambing (167 ± 1.5 bpm) then decreased at 3 weeks before lambing (139.0 ± 15.7 bpm) and reached 117.0 ± 9.2 bpm at birth. In addition, a significant correlation was found between fetal heart rate and gestational age.

F. Placentome size

Placentomes could be detected by transrectal ultrasonography (5 MHz) at Day 30 (Buckrell et al., 1986) and at Day 32 of gestation (Doize et al., 1997). At this period the placentomes appeared as echogenic areas on the surface of endometrium. At Day 42, the ovine placentomes presented cup-shaped forms and reached the maximum size by Day 74 (Doize et al., 1997). There was a poor correlation between placentome size and ovine gestational age due to great variation in the size of placentome in the same observations (Doize et al., 1997; Gonzalez et al., 1998). In contrast, Kelly et al. (1987) found a significant quadratic relationship between ultrasonographic cotyledon diameter and square root transformation of day of pregnancy.

G. Other fetal structures

There was a high correlation ($r = 0.96$) between the width of three ovine fetal coccygeal vertebrea and gestational age. At the same time, somewhat lower correlation was found for umbilical cord diameter ($r = 0.72$) and fetal femur length ($r = 0.78$) (Gonzalez et al., 1998).

5.4. Determination of fetal sex

Depending on the location of the genital tubercle of the ovine fetus, the accuracy of the transrectal ultrasonography (5 MHz) for detecting male and female fetuses was 100% and

76%, respectively from Days 60 to 69 of gestation (Coubrough and Castell, 1998).

CONCLUSIONS

Early detection of pregnancy and determination of the fetal numbers have economical benefits to sheep producers. The method used for pregnancy diagnosis should be simple, accurate, rapid, inexpensive, practical and safe for both operators and animals. Accurate pregnancy diagnosis can be achieved by progesterone and ovPAG or ovPSPB assays, however, their accuracy for differentiating single and multiple fetuses would not be regarded as sufficiently high to be of practical value and they are expensive. Rectal abdominal palpation is a simple, cheap and quick method, however its accuracy for determining multiple pregnancies is low and it may cause abortion or rectal perforation. Doppler technique requires great skill to achieve high accuracy for prediction of fetal numbers. Radiography and transabdominal B-mode ultrasonography accurately diagnose both pregnancy and fetal numbers, but the second technique is cheaper than the first one and has the advantages of being safe and able to detect the fetal viability. The optimum time for using transabdominal or transrectal ultrasonography in sheep ranges from 25 to 100 days of gestation.

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CHAPTER 2

EARLY PREGNANCY DIAGNOSIS IN SHEEP BY PROGESTERONE AND PREGNANCY-ASSOCIATED GLYCOPROTEIN TESTS

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Theriogenology

2003, **59**:1941-1948

ABSTRACT

The aim of this study was to compare the accuracy of the progesterone (P4) and pregnancy associated glycoprotein (PAG) tests for determination of early pregnancy in sheep. Estrus was synchronized in 182 Awassi x Merino ewes and blood samples were collected at Days 0 (day of the insemination), 18, 22, 29, 36, and 50 after artificial insemination (AI). Plasma P4 concentrations at Days 0 and 18 were determined by double antibody radioimmunoassay, while PAG concentrations at Days 22, 29, 36 and 50 were determined by a heterologous, double-antibody radioimmunoassay (RIA) using the bovine PAG 67 kDa subunit as tracer and standard and rabbit antiserum raised against a mixture of caprine 55 and 59 kDa PAG subunits as the first antibody. The discriminatory value for diagnosis of pregnancy by the P4 and the PAG-RIA tests was ≥ 1 ng/ mL. Based on lambing data, the accuracy for diagnosing pregnant (sensitivity) and non-pregnant ewes (specificity) and predictivity of both tests were calculated. The sensitivity, specificity, positive and negative predictive values for P4 and PAG tests were 100 %, 95.4 %, 81.5 %, and 100 % at Day 18 (P4) and 93.5 %, 100 %, 100 % and 98.7 % at Day 22 (PAG), respectively. For diagnosis of non-pregnant ewes the PAG test had significantly higher specificity than the P4 test ($P < 0.05$). It is concluded that ovine pregnancy can be reliably diagnosed at Day 22 after AI by using a heterologous radioimmunoassay of PAG.

Keywords: pregnancy diagnosis; P4; PAG; ewe

INTRODUCTION

Early pregnancy diagnosis is a useful management tool in the sheep industry. Separation of the sheep flock into pregnant and non-pregnant ewes allows better control of management and improved nutrition for the pregnant animals.

There are several methods for pregnancy diagnosis in sheep (1,2,3,4), but only a few methods are useful in detecting early pregnancy. Based on the presence of early pregnancy factor (EPF) in the serum of pregnant ewes, pregnancy can be detected as early as 24 h after fertilization by a rosette inhibition test (5). However, the test is too complex to be applied in the field (3). Real-time transrectal ultrasonography (5MHz) can detect ovine embryonic vesicles as early as Days 17 to 19 after breeding (6), but the technique has a very low sensitivity (12 %) before Day 25 of gestation (7), increasing to 85 % only at Days 32 to 34 of gestation (6). Similarly, transabdominal ultrasonography can provide accurate pregnancy diagnosis only from Day 40 of gestation (8). In contrast, assessment of progesterone (P4) concentration at Days 16 to 18 after mating or AI of sheep is recommended as an early pregnancy test with high (88 % to 100 %) sensitivity. However, the specificity of the test for nonpregnant ewes is variable (60 % to 100 %) (9,10,11).

Pregnancy-associated glycoproteins (PAG) and/ pregnancy-specific protein B (PSPB) belong to the aspartic proteinase family, and are secreted by the trophoblastic binucleate cells (12). They are detectable in the maternal blood around the time of definitive attachment of the fetal placenta when the trophoblastic binucleate cells start to migrate and fuse to the endometrial cells forming the fetomaternal syncytium (13). Therefore these glycoproteins are good indicators of both pregnancy and feto-placental well being. By using heterologous radioimmunoassays, ovPAG and/ or ovPSPB can be detected in the blood of pregnant ewes around Day 20 after mating (14,15,16). Throughout pregnancy, ovPAG concentration varies according to the breed of the ewe and the sex and the number of the fetuses (14,17,18). After lambing, ovPAG and/ or ovPSPB concentrations decrease rapidly, reaching the basal level at week 2 to 4 postpartum (14, 15).

In a field study, the sensitivity and specificity of the heterologous radioimmunoassay of ovPSPB from Days 26 to 106 of gestation were 100 % and 83 %, respectively (19). The pregnancy associated glycoprotein-radioimmunoassay (PAG-RIA) tests have been successfully used for pregnancy diagnosis in cows and goats (20, 21). To our

knowledge, no attempts have been made to evaluate the accuracy of the PAG-RIA test for early pregnancy diagnosis in sheep. The aim of this field study was to compare the accuracy of PAG and P4 tests for early pregnancy diagnosis in sheep.

MATERIALS AND METHODS

1. Animals and Estrus Synchronization

One hundred eighty two Awassi x Merino ewes (1.6-to -10-year- old) were used in the study. The ewes were housed and managed at a farm in eastern Hungary. In all ewes estrus was synchronized by insertion of intravaginal sponges containing 30 mg flurogestone acetate (Chrono-gest, Intervet International B.V. Boxmeer, The Netherlands), for 14 d at the beginning of the breeding season (August). At the time of sponge removal the ewes were administered eCG (600 IU, i.m., Folligon, Intervet). All ewes were inseminated twice with fresh semen at 48 and 56 h after sponge removal. The day of insemination was considered as Day 0 for calculating the gestational period. All ewes were examined for pregnancy by real-time, B-mode ultrasonography (Aloka SSD-500, Aloka Co. Ltd., Tokyo, Japan) on Day 80 after AI.

2. Blood Sampling

Blood samples were collected from each ewe at Days 0, 18, 22, 29, 36 and 50 after AI. Blood samples (5 mL) were withdrawn from the jugular vein into heparinized vacutainer tubes, which were put into a cool box until centrifugation. The plasma was separated within 3 hours after collection by centrifugation at 1500 x g for 20 min, and then stored at -20℃ until assayed for progesterone and PAG concentrations.

3. PAG and P4 Radioimmunoassays

Concentrations of PAG at Days 22, 29, 36 and 50 after AI were detected by a heterologous double-antibody RIA test using the boPAG 67 kDa subunit as tracer and standard, and rabbit antiserum raised against a mixture of caPAG 55 and 59 kDa subunits (R708) as the first antibody. The purified boPAG 67 KDa subunit was radiolabelled by chloramine T using ^{125}I (22). The antiserum used in this assay has been proved to be specific for PAG molecules against other members of the aspartic proteinase family (pepsinogen, pepsin, chymosin, rennet, cathepsin D and renin) (23, in press). Inhibition of binding of the tracer to the antiserum was observed with the sera of the pregnant ewes, while it was not observed with the sera of nonpregnant ewes. Therefore the assay can detect pregnancy in sheep. However, the inhibition curve generated by dilutions of the serum of pregnant ewes was not parallel to the standard

curve. Thus the assay gave relative PAG concentrations which were used to differentiate between pregnant and nonpregnant ewes.

The procedures of the assay were similar to those of Perényi et al. (24) who used the same assay for early pregnancy diagnosis in cows. In addition, the validation and the criteria of the assay have been described by Perényi et al. (23, 24). Briefly, pure stock of the standard was diluted with Tris buffer of pH 7.5 (0.025 M Tris, 0.01 M $MgCl_2$, 0.1% BSA and 0.01% neomycin sulfate) to match the concentrations of the standard curve (from 0.2 ng/mL to 25 ng/mL). The standards and plasma samples (0.1 mL) were diluted with 0.2 mL of Tris buffer. To minimize non-specific interference due to plasma proteins, 0.1 mL of PAG-free sheep serum was added to the standard curve tubes. The antiserum (0.1 mL) was added to all tubes and they were incubated overnight at room temperature. The following day, the tracer (0.1mL, ~28000 cpm) was added to all tubes and they were further incubated for 4 h at room temperature. The purpose of this delayed addition of the tracer is to increase the sensitivity of the assay. One mL of the second antibody polyethyleneglycol (PEG) solution (0.17 % normal rabbit serum, 0.83 % sheep anti-rabbit IgG, 0.4 % BSA, 0.05 % cellulose and 4 % PEG 6000 in Tris buffer) was added to all tubes to facilitate separation of free and bound fractions by centrifugation. After the tubes had been incubated for 1 h, 3 mL of Tris buffer was added to all tubes and they were directly centrifuged at 1500 x *g* for 20 min (at 10˚C). The supernatant was removed by aspiration and the radioactivity of the sediment was counted by using a gamma counter (LKB Wallace 1261 Multigamma counter, Turku, Finland) with a counting efficiency of 75 %. Because of high levels of ovPAG at Days 36 and 50 of gestation, the samples of pregnant ewes at these times were re-assayed without preincubation of the antiserum. The standard curve ranged from 0.8 to 100 ng/mL.

Progesterone concentrations at Day 0 and Day 18 after insemination were detected by double-anitbody radioimmunoassay according to Ranilla et al. (14). The cut-off value of both PAG and P4 assays to diagnose pregnant ewes was ≥ 1 ng/mL.

4. *Analysis of data*

Data for both assays were arranged as follows: correct positive diagnosis (a), incorrect positive diagnosis (b), correct negative diagnosis (c), and incorrect negative diagnosis (d). From these data, the sensitivity (100 x a/a+d), the specificity (100 x c/c+b), the

positive predictive value (100 x a/a+b) and the negative predictive value (100 x c/c+d) of both tests were calculated. The number of animals decreased throughout the study period because some non-pregnant ewes returned to estrus and were re-inseminated. In addition, two pregnant ewes were missed for blood sampling at Day 50 after AI.

The exact binomial test was used to compare the sensitivity and the specificity of the P4 test at Day 18 with the PAG test at Days 22, 29, 36 and 50 after AI by using software package S-Plus 2000 professional edition (Math Soft Int., Knightway House, Park Street, Bagshot, Surrey, GU195AQ, UK). Differences between pregnant and non-pregnant ewes in the level of P4 and ovPAG were statistically analyzed using a Student's *t*-test (25).

RESULTS

The pregnancy rate detected by ultrasonography 80 d after AI was low (31/182). After 80 d three pregnant ewes aborted and 28 lambed after a normal pregnancy length. The average gestation period of these ewes was 150 ± 2.0 d. The accuracy of progesterone and PAG tests for diagnosing pregnancy are shown in Table 1. The sensitivity of the PAG test was high at Day 22 of gestation; only two pregnant ewes had PAG levels lower than 1 ng/mL (false negative diagnoses) at Day 22. From Day 29 onward, the sensitivity reached 100 % accuracy. The specificity of the test was very high (100%) from Day 22 onward; only one false positive diagnosis was made at Day 29. This ewe had a relatively high PAG level (0.8 ng/mL) at the day of insemination.

Regarding the progesterone test, seven non-pregnant ewes had progesterone levels higher than 1 ng/mL (false positive diagnoses) and two had high progesterone level (>1ng/mL) at the day of insemination.

There were no significant differences in sensitivity of the P4 and PAG tests, but the PAG test had a significantly higher specificity ($P < 0.05$) at Days 22, 36, and 50 than that of the P4 test at Day 18 after AI (Table 1).

The P4 and ovPAG concentrations (ng/mL) for both pregnant and non-pregnant ewes are shown in Table 2. The pregnant and nonpregnant ewes showed highly significant differences ($P < 0.0001$) in level of P4 at Day 18 and ovPAG at Days 22, 29, 36 and 50 of gestation.

DISCUSSION

The present study seems to be the first on evaluation of the accuracy of a PAG-RIA test for early pregnancy diagnosis in sheep. Because the ovPAG has not been purified to homogeneity (12), and PAGs from different ruminants are similar in their identity sequences and immunoreactivity (26, 27), a heterologous radioimmunoassy was used. Bovine PAG 67 KDa subunit was used as tracer and standard, while a mixture of caPAG 55 and 59 KDa subunits was used for production of the antiserum.

In the present study, the number of ewes which became pregnant after insemination following synchronized estrus was low (17 %). Similar results were obtained following artificial insemination or natural mating of ewes in synchronized estrus during or at the end of the breeding season (6, 28). As a result, the ability of this study to evaluate positive predictive value was somewhat limited.

The sensitivity and the specificity of PAG tests obtained in this study at Day 22 were high (93.5 % and 100 %); only 2 false negative diagnoses were made. Similar results were obtained using the same assay in goats (21). In contrast, Ranilla et al. (14) reported that detectable levels of PAG were found only in 66.6 % of pregnant Churra and Merino sheep at Day 21 after AI, which is much lower than our results. The sensitivity was 100 % from Day 29 onward and only one false positive diagnosis was made by the PAG-RIA test. The concentration of ovPAG of that ewe at Day 29 was much lower than those of pregnant ewes at the same stage of gestation (1.02 ng/mL vs. 8.1 ±1.4 ng/mL) (Table 2). In addition, the basal value of ovPAG of this ewe at Day 0 was relatively high (0.8 ng/mL), suggesting the presence of PAG molecules originating from extra-uterine tissues as hypothesized by Zoli et al (29). The sensitivity and specificity of the PAG-RIA test obtained in this study at Day 29 after AI are similar to those obtained in goats (21). Generally, the accuracy of the PAG-RIA test of our study from Day 22 onward was higher than those of the heterologous radioimmunoassay of ovine pregnancy-specific protein (ovPSPB) during Days 26 to 106 of gestation (19).

The sensitivity (100 %) and specificity (95.4 %) of the P4-RIA test on Day 18 after AI in our study were higher than those reported by others (10, 30, 31), and similar to those obtained by McPhee and Tiberghien (9). Seven false positive diagnoses were made using the P4 test. Two of these animals had high (> 1 ng/mL) P4 concentration at the day of insemination indicating that these ewes were not in estrus at the time of

insemination. The other five false positive diagnoses may be due to irregularity of the estrous cycle, early embryonic death, pyometra or luteal cysts (2, 32). Thus, assessment of P4 concentration at the day of insemination and Day 18 after breeding may reduce the number of false positive diagnoses (11).

The PAG-RIA test at Days 22, 36 and 50 of gestation had significantly higher specificity for diagnosing nonpregnant ewes than the P4 test. This may be attributable to the fact that PAG is produced by trophoblastic binucleate cells and a level higher than the threshold (≥ 1 ng /mL) is a strong indication of viable trophoblastic tissues. In contrast, P4 levels higher than the threshold (≥ 1 ng /mL) indicate a functional corpus luteum which is associated with pregnancy, natural estrous cycles or ovarian or uterine pathology. The significant difference in specificity between P4 and PAG tests is also a function of the large number (percentage) of non-pregnant ewes. If most ewes were pregnant, a 5 % difference between P4 and PAG tests in detection of nonpregnant ewes would not have been statistically significant. Conversely, the difference between the tests would likely have been significant for sensitivity.

The advantage of the PAG test is that it requires a single plasma sample for early pregnancy diagnosis. In addition, the accuracy of the test for determining pregnancy as early as Day 22 in the present study is higher than those reported for ultrasonography during the first 30 d of gestation. The potential use of the PAG test in a modified kit form will enable sheep producers to apply the test in the field, thus overcoming lab expenses, the radioactivity hazard of using the RIA method and delays in obtaining test results.

In conclusion, the heterologous PAG-RIA test is a reliable method for diagnosing pregnancy in sheep with high accuracy from Day 22 after AI. The advantage of the PAG test over the P4 test is that it can differentiate between pregnancy and prolonged inter-estrus intervals.

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Table I

Sensitivity, specificity and predictive values of the progesterone (P4) and pregnancy-associated glycoprotein (PAG) tests in Awassi x Merino ewes.

Days of pregnancy	Ewes *(n)*	Pregnancy test	a	b	c	d	Se %	Sp %	+PV %	-PV %
18	182	P4	31	7	144	0	100	95.4	81.6	100
22	182	PAG	29	0	151	2	93.5	100 *	100	98.7
29	156	PAG	31	1	124	0	100	99.2	96.9	100
36	155	PAG	31	0	124	0	100	100 *	100	100
50	148	PAG	29	0	119	0	100	100 *	100	100

a: correct positive diagnosis (pregnant), b: incorrect positive diagnosis (nonpregnant), c: correct negative diagnosis (nonpregnant), d: incorrect negative diagnosis (pregnant), Se: sensitivity, Sp: specificity, +PV: positive predictive value, -PV: negative predictive value.

* Significant differences between PAG and P4 tests ($P < 0.05$).

Table II

Concentrations (mean ± SD) of progesterone (P4) and ovine pregnancy-associated glycoprotein (ovPAG) (ng/mL) in pregnant and nonpregnant ewes.

Pregnancy status	P4 (ng/mL)		ovPAG (ng/mL)			
	Day of sampling		Day of sampling			
	0[1]	18	22	29	36	50
Non-pregnant (*n*)	0.2 ± 0.3 (151)	0.4 ± 0.4 [b] (151)	0.2 ±0.1 [b] (151)	0.2 ± 0.1 [b] (125)	0.4± 0.2 [b] (124)	0.3 ± 0.2 [b] (119)
Pregnant (*n*)	0.2 ± 0.1 (31)	3.3 ± 0.9[c] (31)	4.3 ± 1.4[c] (31)	8.1 ± 1.4 [c] (31)	26.1 ± 8.2 [c] (31)	30.2 ±10.9 [c] (29)

Different superscripts (a and b) in the same column indicate a significant ($P < 0.0001$) difference between pregnant and non-pregnant ewes.

[1]Day of insemination.

CHAPTER 3

ACCURACY OF TRANSRECTAL ULTRASONOGRAPHY FOR DETERMINATION OF PREGNANCY IN SHEEP: EFFECT OF FASTING AND HANDLING OF THE ANIMALS

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Theriogenology
2003 (in press)

ABSTRACT

The present study was performed to investigate the effect of previous fasting and lifting of the abdomen of the ewes during transrectal ultrasonographic scanning on the results of early pregnancy diagnosis.

Ewes of four flocks (A, B, C and D; all Awassi x Merino ewes, n= 1247) aged 0.7 to 10 years were used in this study. These ewes were estrus synchronized and artificially inseminated. From two weeks later onwards, fertile rams were kept with the ewes of flocks A, B and C (n=949) for natural breeding, while ewes of flock D (n=298) were re-inseminated 17 days later. Transrectal ultrasonography (5 MHz) was carried out in ewes of flocks A, B and C on four separate occasions but only once in ewes of flock D. For final analysis, animals were divided over two groups: ewes of Group 1 (n=949 scans) were scanned in a standing position within the milking parlor. Animals of Group 2 (n=764 scans) were scanned by the same operator and with the same scanning technique, but these ewes were fasted for 12 hours prior to scanning and the abdominal wall was lifted, just in front of the udder during scanning.

The sensitivity of the test for diagnosing pregnancy at Days 18 to 24, 25 to 30, 31 to 40 and 41 to 50 was 21.8 %, 32.3 %, 63.3 % and 50 % in Group 1, and 46 %, 92.5 %, 92.3 % and 96.8 % in Group 2, respectively. Only within Group 1, the sensitivity of the test was higher in young ewes (0.7 to 2 years) than in older ones (>2 to 10 years). Significant differences were observed at scan periods Days 18 to 24 and Days 41 to 50 of gestation. It is concluded that, fasting prior to scanning and lifting the abdomen during scanning significantly improve the accuracy of transrectal ultrasonographic pregnancy diagnosis in Awassi x Merino ewes.

Keywords: transrectal ultrasonography; pregnancy diagnosis; sheep

INTRODUCTION

Early and accurate diagnosis of pregnancy plays an important role in reproductive management on sheep farms. Early identification of non-pregnant ewes allows breeders to apply the various methods to increase the number of the pregnant animals, especially after out-of- season breeding or the using of artificial insemination (AI). Among the numerous methods that have been used for pregnancy diagnosis in sheep, real-time B-mode ultrasonography was proved to be an accurate method that can easily be used on the farm. Between Days 40 to 90 of gestation transabdominal scanning accurately determines both the presence or absence of a pregnancy and the number of the fetuses present in *utero* (1,2,3). However, in many breeds this technique requires the ventral part of the abdomen to be shaved, which may be time-consuming and laborious in large-scale farms. During transrectal scanning with a 5 MHz transducer, early pregnancy can be recognized already at Days 17 to 19 by the presence of anechoic fluid in the uterus (4), while the embryo proper and placentomes can be visualized on Days 26 to 28 of gestation (5). However, the accuracy of transrectal scanning with a 5-MHz transducer for the detection of pregnancy between Days 17 and 50 varies greatly (4, 5, 6, 7). The age and the breed of the ewes and the experience of the operator are among the main factors responsible for these variable results (8,9). To our knowledge, the factors affecting the accuracy of transerctal ultrasongraphy for the detection of early pregnancy have not been investigated in sheep. Therefore, the present field study was performed to investigate the effect of fasting prior to scanning and lifting of the abdomen during transrectal scanning on the accuracy of pregnancy diagnosis.

MATERIALS AND METHODS

1. Estrus synchronization and breeding of the ewes

Four flocks (A, B, C and D) of Awassi x Merino ewes (*n*= 1247), aged 0.7 to 10 years, were used in this study. These ewes were housed and managed on a commercial farm in eastern Hungary. Estrus was synchronized by means of intravaginal sponges (each containing 30-mg flurogestone acetate; Chrono-gest, Intervet International B.V., Boxmeer, The Netherlands) installed for 14 days during the second half of August 2000. At the time of sponge removal, each ewe was treated (i.m.) with eCG (600 IU, Folligon, Intervet International B. V.). All ewes were inseminated, deep into vagina, twice with fresh semen at 48 and 56 h after sponge removal. The day of insemination was considered as Day 0 for calculating the gestational age. Two weeks after AI, fertile rams were introduced to flocks A, B and C to mate those ewes returned to estrus, while all ewes of flock D (n=298) were re-inseminated on Day 17. Because the exact dates of breeding of the naturally mated ewes were unknown, gestational ages at the time of scanning of these ewes were calculated retrospectively by subtracting the number of days elapsing between scanning and lambing from the average duration of gestation (150 days) of this breed (10).

2. Ultrasonographic examinations

A real-time ultrasound scanner equipped with a 5 MHz linear-array transducer (Aloka SSD-500, Aloka Co. Ltd., Tokyo, Japan) was used for this study. The transducer was taped to a plastic rod to allow manipulation from the outside after it had been inserted into the rectum (4). All the ultrasonographic examinations were done by the same operator who had been experienced in performing ultrasonographic pregnancy diagnosis in small ruminants. Transrectal scanning were performed on ewes of flocks A, B and C on four separate occasions. At the first and second occasions, the ultrasound scanning was carried out on all ewes. There was an interval of 40 days between the first and second occasions. The ewes which had been diagnosed as pregnant were separated, the non-pregnant ewes remained kept with the fertile rams and scanned again on two separate occasions, this time at an interval of 33 days. Ewes of flock D (*n* =298) were scanned only once between Days 18 to 39 after AI. For final analysis of the data, the results of ultrasound scanning (n=949 scans) carried out on the first occasion on ewes of flocks A, B and C were attributed to Group 1. Ewes belonging to this group were

restrained in a standing position during scanning in the milking parlor. If necessary the rectum was cleared of feces and the lubricated transducer was gently inserted into the rectum till the anechoic content of the urinary bladder became visible. Then it was rotated clockwise 90° and anti-clockwise 180° to scan the entire reproductive tract (11). The scans (*n*=764) made during the second, third and fourth occasions on ewes of flocks A, B and C and all ewes of flock D were attributed to Group 2. Ewes belonging to this group were scanned by using the same technique as applied in the animals of Group 1, but they had been fasted for 12 h prior to scanning. In addition, the ventral abdominal wall in front of the udder of the ewes was lifted up by the assistant's hands while conducting the scanning. Depending on the stage of pregnancy, the recognition of the allantoic fluid, embryo proper, placentomes, or a fetus were considered as positive signs of pregnancy in both groups.

3. Analyses of data

Inseminated, non-lambing ewes were distributed over the gestational ages at scanning according to their dates of AI known for Group 1 and flock D (Group 2). Non-lambing ewes, which had been mated, were distributed over different gestational ages at scanning according to a ratio related to the stage of gestation of the animals, which lambed in Group 2 (3).

To study the influence of the age of ewes on the accuracy of transrectal ultrasonography, each of the two groups was subdivided into two subgroups; the first included young ewes (aging 0.7 to 2 year), which were primiparious, or nulliparous. The second subgroup included ewes aged between more than 2 to 10 years and were pluriparous.

Based on lambing performance of the tested ewes, the results of ultrasonographic examinations were arranged as follows: correct positive diagnosis (a), incorrect positive diagnosis (b), correct negative diagnosis (c), and incorrect negative diagnosis (d). From these values the sensitivity (a/a+d x 100), the specificity (c/c+b x 100), the positive predictive value (a/a+b x 100) and the negative predictive value (c/c+d x 100) of the test in Groups 1 and 2 and their subgroups were calculated (12)**.**

The sensitivity and specificity of transrectal ultrasonogrpahy obtained in Groups 1 and 2 and in their subgroups were compared by Fisher's exact test (total number of the ewes <

200) and Pearson's Chi-square with Yate's continuity correction (total number of the ewes > 200) using software package S Plus 2000 professional (Math Soft Int., Park St. Bagshot, Surrey GUI 195AQ, UK). These statistical tests were also used to test if the sensitivity and specificity of the technique differed significantly between different days of pregnancy within each group. Due to the multiple comparisons in the sensitivity and specificity of the technique between days of pregnancy in each group, Bonferroni's correction was applied (13).

RESULTS

A total of 1713 ultrasound scans were made in 1247 ewes. The accuracies of pregnancy diagnosis in Groups 1 and 2 are shown in Table 1. The sensitivity (the accuracy for detecting pregnant ewes) of the test increased when performed at a more advanced stage of pregnancy, reaching a maximum of 63.3 % at Days 31 to 40 in Group 1 and a maximum of 96.8 % at Days 41 to 50 in Group 2. In Group 1, significant differences in the sensitivity of the test were observed between scan periods Days 25 to 30 and Days 31 to 40 (P= 0.003) and between scan periods Days 18 to 25 and Days 31 to 40 (P< 0.0001) and 41 to 50 (P= 0.002) (Table 1). In Group 2, the significant differences (P<0.0001) were observed between scan periods Days 18 to 24 and other periods of examination (Days 25 to 30, 31 to 40, and 41 to 50) (Table 1). Furthermore, the sensitivities of the tests performed in Group 2 were significantly higher than those of the tests in Group 1 during all periods of the scanning (Table 1). On the other hand, the specificities of the test (accuracy of detecting non-pregnant ewes) were always high in both groups. No significant differences in the specificity of the test were observed between the two groups with the exception of scan periods Days 41-50 during which the specificity of the test in Group 1 was significantly (P< 0.01) higher than that of Group 2 (Table 1).

A total number of 74 (47 in Group 1 and 27 in Group 2) false positive pregnancy diagnoses were made during the examinations performed between Days 18 to 50 of gestation.

Regarding the effect of the age of the ewes, the sensitivity of the test was significantly higher in young ewes of Group 1 at scan periods Days 18 to 24 and Days 41 to 50 of gestation (Table 2). In addition, the sensitivity of the test increased in young ewes when scanning took place at a more advanced stage of pregnancy, reaching the maximum at scan period Days 41 to 50. By contrast, in older ewes the sensitivity of the test decreased after Day 40 of gestation (Table 2). The accuracies of the test in the two age subgroups of Group 2 were not significantly different (Table 3).

DISCUSSION

During early pregnancy (Days 18 to 20), the ovine conceptus has a filamentous structure with small amount of fluid occupying both uterine horns and the uterine body (5). During ultrasonographic scanning this fluid appears as circular or elongated anechoic areas about 4 mm in diameter (4). Before Day 25 of gestation it may be difficult to identify the conceptus by a 5 MHz linear-array transducer, especially when large-sized, mature ewes are scanned under field conditions (14). This observation was confirmed by the significantly higher sensitivity of the test in young ewes than that in older ones in Group 1 between Days 18 to 24 of gestation. In addition, it may explain the low sensitivity of the test obtained in Group 1 (21.8 %) and Group 2 (46 %) between Days 18 to 24 of gestation. Similarly, low sensitivities (12 % to 53 %) of the test were reported by other studies using transrectal scanning between Days 17 to 24 of gestation (4,6). In contrast, a much higher sensitivity (99 %) of transrectal scanning has been reported in one study between Days 14 to 25 of gestation (7). Possibly breed differences between ewes of the two studies play a dominant role in this respect. The significant increase in the sensitivity of the test in Group 2 between Days 18 to 24 and Days 25 to 30 (46 % vs. 92.5 %, respectively) might be explained by the rapid increase in the volume of the embryonic fluid from Day 25 of gestation onwards, so that a larger portion of the embryonic vesicle becomes more ultrasonographically visible (15). In addition, the embryo proper and placentomes could be imaged between Days 26 and 28 of gestation (5). This significant increase in the sensitivity of the test was observed later in Group 1 between Days 25 to 30 and Days 31 to 40. The overall sensitivity of the test in Group 1 (54 %) between Days 25 to 50 of gestation was low when compared with that of other studies using a 5 MHz linear-array transducer and carried out without fasting and lifting of the abdomen (4, 6, 7). However, the scanning conditions and the age and breed of the animals were different in these studies. Gearhart et al (6) reported a slightly higher sensitivity (64.8 %) of transrectal scanning at Days 26 to 50 of gestation. However, the authors performed serial ultrasonographic examinations at 2 to 3-day intervals on experimental animals *(n* =26) and the ewes were scanned in a lateral recumbent position to retain the reproductive tract close to the pelvis. On the other hand, Garcia et al. (4) reported a much higher sensitivity (75 %) of the test at Days 24 to 34. In that study, 91 nulliparous and pluriparous ewes were scanned repeatedly in a standing position at 4-day intervals from Days 17 to 34 of gestation. In addition, 100 % sensitivity of the test was reported in one study at Days 25 to 29 of gestation (7). In that

study 1442 German Merino-Mutton ewes (small-sized breed compared to the breed of our study) were scanned in a standing position under field conditions. The sensitivity (94 %) of the test in Group 2 of our study between Days 25 to 50 was higher than that (87 %) reported by Buckrell et al. (5). In that study, mature ewes (n=64) were scanned in a standing position first at 25 to 30 days after breeding and the scanning was repeated at 5 day-intervals up to Day 50 of gestation if the diagnosis was one of not pregnant. The animals were fasted for 12 hours before scanning in both experiments, but the abdomen of the ewes was lifted up only in our study.

In Group 1 significantly lower sensitivities of the test were obtained than in Group 2 at all periods of examination. This may be explained by the fact that in this type of breed used in the present study, the pregnant uterus tops over the pelvic brim and descends into the abdominal cavity at an early stage of pregnancy, especially in large sized pluriparous ewes (8, 14). This effect was obviously overcome by lifting up the abdomen of ewes in the Group 2. In addition, fasting the animals prior to scanning minimizes the presence of intestinal gases, which might obscure the imaging of the reproductive tract and consequently increases the occurrence of false negative diagnosis (14). This hypothesis of an earlier abdominal descend of the pregnant uterus in mature pluriparious ewes was supported by the higher sensitivity of the test in young ewes of Group 1 between Days 18 to 50 of gestation. In addition the sensitivity of the test decreased in older ewes after Day 40 of gestation, while it still increased in young ewes in the same period.

The specificity of the test was similarly high in both groups, with the exception of examinations performed during the scan period Days 41 to 50. A significantly higher specificity of the test was obtained in Group 1 than that in Group 2, but this might be largely influenced by the rather small number of non-pregnant ewes in Group 2. Similar high specificities of the transrectal ultrasonographic examination have been reported (95 % to 98 %) by the others (4,5). However, a lower specificity of the test was obtained in one study, in which the number of non-pregnant ewes was relatively small (7). The most likely source of false positive diagnoses might be embryonic mortality or abortion, which may reach to 30 % in sheep (16). In addition, abnormal uterine conditions like pyometra or hydrometra or the presence of intestinal or abdominal fluid may lead to false positive diagnosis, especially in the early stage of pregnancy. Further studies are

required to differentiate between the above mentioned possibilities of the false positive diagnoses.

In summary, the age of the Awassi x Merino ewes has an effect on the accuracy of transrectal ultrasonography for detection of pregnancy. In addition, fasting Awassi x Merino ewes for 12 hours prior to scanning and lifting up their abdomen while conducting the scanning significantly improve the accuracy of the transrectal ultrasonography for detecting early pregnancy.

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Table 1

Sensitivity (Se), specificity (Sp), and predictive values (+PV, -PV) of transrectal ultrasonography for pregnancy diagnosis in Groups 1 and 2 of Awassi x Merino ewes

Grouping and evaluation	Group 1 (n=949) Days of gestation 18-24 (n=161)	25-30 (n=84)	31-40 (n=352)	41-50 (n=352)	Group 2 (n=764) Days of gestation 18-24 (n=113)	25-30 (n=228)	31-40 (n=248)	41-50 (n=175)
a	14	11	69	28	23	99	109	123
b	7	4	20	16	5	7	7	8
c	90	46	223	280	58	114	123	40
d	50	23	40	28	27	8	9	4
Se (%)	21.8 $^{e\ IK}$	32.3 $^{g\ J}$	63.3 $^{gI\ J}$	50.0 $^{g\ K}$	46.0 $^{f\ L}$	92.5 $^{h\ M}$	92.3 $^{h\ M}$	96.8$^{h\ M}$
Sp (%)	92.7	92.0	91.7	94.6 ^{e}	92.1	94.2	94.6	83.3^{f}
+PV (%)	66.6	73.3	77.5	63.6	82.1	93.3	93.9	93.8
-PV (%)	64.2	66.6	84.7	90.9	68.2	93.4	93.1	90.9

a: correct positive diagnosis (pregnant), b: incorrect positive diagnosis (non pregnant), c: correct negative diagnosis (non-pregnant), d: incorrect negative diagnosis (pregnant).

$^{e, f}$ (P<0.01) , $^{g, h}$ (P< 0.0001): significant difference between the two groups.

Percentages with the same capital letter superscript in Group 1 are significantly different (P<0.0001, P= 0.003, P=0.002 for I, J and K respectively).

$^{L, M}$ (P< 0.0001): significant difference within Group 2.

Figures in parentheses indicate the numbers of ultrasonographic examinations**.**

Table 2

Effect of age of ewes on the accuracy of transrectal ultrasonography for pregnancy diagnosis (Group 1)

Days of gestation	Age of ewes (year)	No. of ewes	a	b	c	d	Se %	Sp %	+PV %	-PV %
18-24	0.7 to 2	54	9	4	35	6	60.0 [e]	89.7	69.2	85.3
	>2 to 10	107	5	3	55	44	10.2 [f]	94.8	62.5	55.5
25-30	0.7 to 2	46	4	2	38	2	66.6	95.0	66.6	95.0
	>2 to 10	38	7	2	8	21	25.0	80.0	77.7	27.5
31-40	0.7 to 2	83	17	3	56	7	70.8	94.9	85.0	88.8
	>2 to 10	269	52	17	167	33	61.1	90.7	75.3	83.5
41-50	0.7 to 2	60	7	2	51	0	100 [g]	96.2	77.7	100
	>2 to 10	292	21	14	229	28	42.8 [h]	94.2	60.0	89.1

a: correct positive diagnosis (pregnant), b: incorrect positive diagnosis (non pregnant), c: correct negative diagnosis (non-pregnant), d: incorrect negative diagnosis (pregnant). Se: sensitivity, Sp: specificity, +PV: positive predictive value, -PV: negative predictive value.

[e, f] $P<0.001$.

[g, h] $P<0.05$.

Table 3

Effect of age of ewes on the accuracy of transrectal ultrasonography for pregnancy diagnosis (Group 2)

Days of gestation	Age of ewes (year)	No. of ewes	a	b	c	d	Se %	Sp %	+PV %	-PV %
18-24	0.7 to 2	14	3	1	3	7	30.0	75	75.0	30.0
	>2 to 10	99	20	4	55	20	50.0	93.2	83.3	73.3
25-30	0.7 to 2	49	26	3	19	1	96.2	86.3	89.6	95.0
	>2 to 10	179	73	4	95	7	91.2	95.9	94.8	93.1
31-40	0.7 to 2	32	19	1	11	1	95.0	91.6	95.0	91.6
	>2 to 10	216	90	6	112	8	91.8	94.9	93.7	93.3
41-50	0.7 to 2	38	31	2	4	1	96.8	66.6	93.9	80.0
	>2 to 10	137	92	6	36	3	96.8	85.7	93.8	92.3

a: correct positive diagnosis (pregnant), b: incorrect positive diagnosis (non pregnant), c: correct negative diagnosis (non-pregnant), d: incorrect negative diagnosis (pregnant). Se: sensitivity, Sp: specificity, +PV: positive predictive value, -PV: negative predictive value.

Chapter 4

EVALUATION OF FALSE TRANSRECTAL ULTRASONOGRAPHIC PREGNANCY DIAGNOSES IN SHEEP BY MEASURING PLASMA LEVEL OF PREGNANCY-ASSOCIATED GLYCOPROTEINS

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Reproduction, Nutrition, Development
2003 (in press)

ABSTRACT

The present study was undertaken to investigate to what extent pregnancy diagnoses made by transrectal ultrasonography could be confirmed by measurements of plasma concentration of ovine pregnancy-associated glycoproteins (ovPAG). A total of 424 Awassi x Merino ewes were synchronized for estrus and examined by transrectal ultrasonography. In Experiment 1, ewes (*n*=156) were repeatedly scanned in a standing position at Days 29, 36 and 50 of gestation. Similarly, ewes (*n*=268) in Experiment 2 were scanneDays at Days 24, 29 and 34 of gestation, but these ewes were fasted for 12 hours prior to the examination and the abdominal wall of each animal was lifted up by the hands of the assistant during scanning. Blood samples were withdrawn after each transrectal ultrasonographic examination in both experiments. Ovine PAG concentrations were measured in plasma by a heterologous radioimmunoassay and the cut-off value for pregnancy was ≥1 ng/mL. Based on the lambing performance, in Experiment 1, altogether 47 false negative and 38 false positive diagnoses were made by transrectal_ultrasonography in 24 and 33 ewes, respectively between Days 29 and 50 of gestation. In Experiment 2, altogether 8 false negative and 13 false positive diagnoses both were made in 7 ewes between Days 24 and 34 of gestation. In both experiments, all ewes with false negative diagnoses had ovPAG concentrations higher than the threshold level for pregnancy diagnosis and all ewes with false positive diagnoses had ovPAG concentrations lower than the threshold of pregnancy. Furthermore, by PAG-RIA test all lambed or aborted ewes (*n*=63) were correctly diagnosed as pregnant and with three exceptions, all non-lambed ewes (*n*=361) were correctly diagnosed as non-pregnant during the examined periods of both experiments.

Keywords: transrectal ultrasonography; ovPAG; pregnancy diagnosis; sheep

INTRODUCTION

Real time B-mode ultrasonography provides a simple, rapid, accurate and non-invasive means for ovine pregnancy diagnosis on the farm [1]. Ovine pregnancy (anechoic intrauterine fluid) can be diagnosed by transrectal ultrasonography (5 MHz) as early as Days 17 to 19 of gestation, while the embryo proper can be imaged between Days 21 to 34 of gestation [2]. However, the accuracy of transrectal ultrasonography (5 MHz) for early pregnancy diagnosis reported in several studies is rather variable. In these studies 1.2 % to 87.2 % of lambing ewes were incorrectly diagnosed as non-pregnant (false negative diagnoses) and 0 % to 35.3 % of non-lambing ewes were incorrectly diagnosed as pregnant (false positive diagnoses) before Day 25 of gestation [2-4]. Even between Days 25 to 50 of gestation, the percentages of false negative (0 % to 35 %) and false positive (0 % to 17.5 %) diagnoses made by transrectal ultrasonography (5MHz) were still conspicuous [2-6]. The variable results obtained in the above mentioned studies might be due to the effect of the breed and the age of ewes [7], whether the ewes were fasted prior to scanning or not [8], the method of handling ewes [8], the experience of the operator [9] and the incidence of embryonic or fetal mortality [7].

Ovine pregnancy-associated glycoproteins (ovPAGs) are members of the aspartic proteinase family [10]. They are synthesized by the superficial epithelial layer (mono – and binucleate cells) of the fetal placenta [11]. Therefore, these glycoproteins are good indicators of a live conceptus [12]. Ovine PAGs are detectable in the maternal blood from the third week of gestation until after lambing. After lambing, plasma ovPAG level decreases rapidly and becomes undetectable during the fourth week postpartum [13]. During pregnancy, the plasma ovPAG concentrations vary according to the breed of the ewe, the stage of pregnancy, and the number and genotype of the fetuses [13,14]. Moreover, the antiserum used in the radioimmunoassay of PAG is a source of variation for PAG concentrations in the same animal [15,16].

In spite of evidence, by molecular cloning studies, that there is expression of many PAG genes in the trophectoderm of ruminant placentas [11,17], few PAG molecules have been isolated and purified. Among these glycoproteins, bovine (bo) PAG 67 KDa [18], ovPAG-1 [19], and caprine (ca) PAGs 55, 59 and 62 KDa [20] are more closely related to each other

both as far as their antigenic properties and their amino acids identities are concerned [10, 20].

The heterologous radioimmunoassay, using boPAG 67 KDa as standard and tracer and antiserum raised against a mixture of ca PAG 55+59 KDa, accurately selected both pregnant (93.5 %) and non-pregnant ewes (100 %) at Day 22 of gestation. The accuracy of the test to select pregnant ewes increased to 100 % at Day 29 of gestation [21]. Because the results of early pregnancy diagnoses obtained using transrectal, real-time, B-mode diagnostic ultrasonography with 5 MHz linear-array transducer are variable, it is necessary to evaluate the false ultrasonographic diagnoses. The present study was undertaken to investigate to what extent pregnancy diagnoses made by transrectal ultrasonography could be confirmed by measurements of plasma concentration of ovPAG on the same day.

MATERIALS AND METHODS

1. Estrus synchronization and breeding of the ewes

A total of 424 Awassi x Merino ewes, aged 1.6 to 10 years, were used in the present study. The ewes were synchronized for estrus by insertion of intravaginal sponges containing 30-mg flurogestone acetate (Chrono-gest, Intervet International B.V., Boxmeer, The Netherlands) for 14 days during the second half of August 2000 (*n*=156) and 2001 (*n*=268), respectively. At the time of sponge removal, each ewe was administered 600 IU eCG (Folligon, Intervet International B. V.) intramuscularly. All ewes were inseminated twice with fresh semen (200 x 10^6 spermatozoa) into the external os of the cervix at 48 and 56 h after sponge removal. The day of insemination was considered as Day 0 for calculating the gestational age.

2. Ultrasonographic examination

2.1. Experiment 1

One hundred and fifty six ewes were scanned on Days 29, 36 and 50 after AI, using a real-time ultrasound scanner equipped with a 5 MHz linear-array transducer (Aloka SSD-500, Aloka Co. Ltd., Tokyo, Japan). The transducer had been modified by taping a plastic rod to the probe to control the manipulation of the transducer inside the rectum. The same operator who had been experienced for ultrasonographic pregnancy diagnosis in small ruminants carried out all the scanning. Ewes were scanned in a standing position in the milking parlor. The rectum was cleared of feces when necessary. The lubricated transducer was gently inserted into the rectum till the urinary bladder could be seen and then it was rotated clockwise 90° and anti-clockwise 180° to scan the entire reproductive tract [22]. Recognition of the allantoic fluid was considered a positive sign of pregnancy. On Day 50, recognition of the fetus(es) or placentomes was used as the criterion for a positive pregnancy diagnosis. All ewes were scanned further by transabdominal ultrasonography at Day 80 of gestation.

2.2. Experiment 2

Two hundred and sixty eight ewes were scanned at Days 24, 29 and 34 after AI by the same operator using the same machine and technique applied in Experiment 1. However, the ewes were fasted for 12 hours prior to scanning and their ventral abdominal wall in front of the udder was lifted up by the hands of an assistant while conducting the scanning. Recognition of the allantoic fluid was considered a positive sign of pregnancy. Ewes diagnosed pregnant

ultrasonographically at Day 34 were scanned further by transabdominal ultrasonography at Days 50 and 80 of gestation.

At each examination in both experiments, the operator was required to record a diagnosis of either pregnancy or non-pregnancy without reference to earlier results. The number of ewes in each experiment decreased during the study periods, whereas some non-pregnant ewes returned to estrus and were re-inseminated. In addition, two pregnant ewes in Experiment 1 were missed at Days 50 of gestation.

3. Blood sampling

After each transrectal ultrasonography, a blood sample (5 mL) was withdrawn from the jugular vein of each ewe into a heparinized vacutainer tube. Immediately (Experiment 2) or within 3 h (Experiment 1) after the collection, blood samples were centrifuged at 1500x *g* for 20 min. The collected plasma was stored at –20 $^{\circ}$C till the assessment of ovPAG.

4. PAG Radioimmunoassay

Concentrations of ovPAG at Days29, 36 and 50 (Experiment 1) and at Days 24, 29 and 34 (Experiment 2) after AI were detected by a heterologous double-antibody RIA test. The boPAG 67 kDa was used as a tracer and standard, while rabbit antiserum raised against a mixture of caPAG 55 and 59 kDa (R708) was used as the first antibody. The antiserum used in this assay has been proved to be specific for PAG molecules against other members of the aspartic proteinase family (pepsinogen, pepsin, chymosin, rennet, cathepsin D and renin) [23]. Inhibition of binding of the tracer to the antiserum was observed with the sera of the pregnant ewes, while it was not observed with the sera of non-pregnant ewes. Therefore the assay can detect pregnancy in sheep. However, the inhibition curve generated by dilutions of the serum of pregnant ewes was not parallel to the standard curve. Thus the assay gave relative PAG concentrations which were used to differentiate between pregnant and non-pregnant ewes [21].

The procedures and the validation criteria of the assay were similar to those of Perényi et al. [24] and were described elsewhere [21]. The cut-off value of PAG-RIA test used to detect pregnant ewes was ≥1 ng /mL [21].

5. Data analysis

Based on the lambing performance or any other observed sign like abortion, the results of transrectal ultrasonographic examinations and the PAG test were arranged as follows: correct positive diagnosis (a), incorrect positive diagnosis (b), correct negative diagnosis (c), and incorrect negative diagnosis (d). From these values the sensitivity (a/a+d x 100), the specificity (c/c+b x 100), the positive predictive value (a/a+b x 100) and the negative predictive value (c/c+d x 100) of both tests were calculated for both experiments [25]. The exact binomial test was used to compare the sensitivity and the specificity of the transrectal ultrasonography with those of PAG test. The same test was also used to compare the sensitivity and specificity of transrectal ultrasonography between days of examinations in each experiment. The age of the ewes with the false negative diagnoses was compared with the age of ewes correctly diagnosed as pregnant by transrectal ultrasonography by means of a Student's *t*-test [26].

RESULTS

The accuracies of the transrectal ultrasonography for ovine pregnancy diagnosis in Experiments 1 and 2 are shown in Tables 1 and 2, respectively.

Experiment I

Twenty-eight ewes lambed with a normal gestation length and 3 ewes aborted (after Day 80) after insemination at the synchronized estrus. All lambed or aborted ewes were diagnosed as pregnant by PAG-RIA at Days 29, 36 and 50. However, by transrectal ultrasonography pregnancy was detected only in 16 ewes (51.6%) at Day 29, 13 ewes (41.9 %) at Day 36 and in 15 ewes (51.7 %) at Day 50 of gestation (Table 1). The level of sensitivity of PAG-RIA test for detecting pregnant ewes was significantly (P<0.001) higher than that of transrectal ultrasonography during each of examination days. Forty-seven false negative diagnoses (15 at Day 29, 18 at Day 36 and 14 at Day 50) were made in 24 ewes by transrectal ultrasonography. Eight of the 24 ewes were diagnosed ultrasonographically as non-pregnant during each of the scanning days. All lambed or aborted ewes without ultrasonographic positive detection (n=24) had a concentration of ovPAG higher than the threshold of pregnancy (Figure 1). At each day of scanning, the mean age of ewes incorrectly diagnosed as non-pregnant by transrectal ultrasonography was significantly (P<0.05 Day 29; P<0.005, Days 36 and 50) higher than that of ewes correctly diagnosed pregnant (Table 3).

With one exception, all non-lambing ewes had ovPAG concentration lower than the threshold of pregnancy at Days 29, 36 and 50 after AI. This ewe had ovPAG concentration slightly higher (1.02 ng/mL) than the threshold of pregnancy at Day 29 and was ultrasonographically diagnosed non-pregnant during each of the scanning days. By transrectal ultrasonography, non-pregnancy was diagnosed in 104 ewes (83.2 %) at Day 29, 110 ewes (88.7 %) at Day 36 and in 116 ewes (97.4%) at Day 50 (Table 1). The level of specificity of the PAG-RIA test was significantly (P<0.001) higher than that of transrectal ultrasonography at Days 29 and 36 of gestation. The level specificity of transrectal ultrasonography at d 50 was significantly higher than that of the test at Days 29 (P= 0.0003) and 36 (P= 0.0127) of gestation (Table 1).

Thirty-eight false positive diagnoses (21 at Day 29, 14 at Day 36 and 3 at Day 50) were made by transrectal ultrasonography in 33 ewes. None of these ewes were incorrectly

diagnosed as pregnant during each of the scanning days. All non-lambing ewes, which ultrasonographically diagnosed pregnant, had oPAG level lower than the threshold of pregnancy.

Experiment 2

Twenty-nine ewes lambed with a normal gestation length and 3 ewes aborted (one after Day 50 and 2 after Day 80 of gestation) after insemination at the synchronized estrus. Similar to Experiment 1, all lambing or aborted ewes had ovPAG level higher than the threshold of pregnancy during the examined periods. However, by transrectal ultrasonography pregnancy was diagnosed in 26 ewes (81 %) at Day 24, 31 ewes at Days 29 and 34 (96.8 %) of gestation (Table 2). The level of sensitivity of PAG-RIA test was significantly (P<0.05) higher than that of transrectal ultrasonography only at Day 24. Eight false negative diagnoses (6 at Day 24, 1 at Day 29 and 1 at Day 34, respectively) were made by transrectal ultrasonography in 7 ewes during the examined period. All ewes with the false negative diagnoses had ovPAG concentration higher than 1 ng/mL (Table 4). With two exceptions, all non-lambing ewes had ovPAG concentrations lower than the threshold level established for pregnancy. The first ewe had high ovPAG concentration (2.3 ng/mL) at Day 24, then the level decreased to 1.5 and 1.2 ng/mL at Days 29 and 34 after AI, respectively. At the same time this ewe was diagnosed non-pregnant by transrectal ultrasonography during the examined periods. The second ewe had ovPAG level (1.16 ng/ mL) slightly higher than the threshold of pregnancy at d 29, while it had undetectable ovPAG levels at Days 24 and 34 of gestation. This ewe was also diagnosed as non-pregnant by transrectal ultrasonography during each of the scanning days.

Thirteen false positive diagnoses (7 at Day 24, 3 at Day 29 and 3 at Day 34, respectively) were made by transrectal ultrasonography in 7 ewes during the study period. These ewes had ovPAG level lower than the threshold of pregnancy (Table 5).

DISCUSSION

In previous studies carried out without fasting or lifting the abdomen of the animals, the accuracy of the transrectal ultrasonography (5 MHz) for diagnosing pregnancy (sensitivity) ranged from 65 % to 100 % between Days 26 to 50 of gestation [2,3,4,6]. A lower sensitivity (48.3 %) of the test was obtained in our Experiment 1 between Days 29 and 50 of gestation. The most probable reasons for this might be the differences in the breed and age of the ewes or in the experience of the operators [7, 9]. Forty-seven false negative diagnoses were made in 24 ewes by transrectal ultrasonography between Days 29 and 50 of gestation. All these ewes had ovPAG concentrations higher than the threshold for pregnancy detection (≥1ng/mL). The sources of these false negative diagnoses might be the early descend of the gravid uterus into the abdominal cavity, especially in the large and pluriparous ewes, then becoming out of reach of the transducer [3, 27]. This hypothesis is supported by the significant higher age of ewes with false negative diagnoses made by transrectal ultrasonography. This source of false negative diagnosis could be less important with transabdominal ultrasonography. Also, in cows the position of the uterus relative to the pelvic inlet has proved to influence the accuracy of transrectal ultrasonograpy for early pregnancy diagnosis [28]. In addition, because the ewes were not fasted prior to scanning, the intestinal gas or ingesta may have interfered with the visualization of the pregnant uterus [3, 27, 29].

The specificity (90 %) of the transrectal ultrasonography for diagnosing non-pregnant ewes obtained in the Experiment 1 between Days 29 to 50 was in the range (82.5 % to 100 %) reported by other studies [2,3,4,6]. Thirty-eight false positive diagnoses were made by transrectal ultrasonography in 33 ewes between Days 29 to 50 of gestation. At the same time, none of these ewes had ovPAG concentration ≥ 1ng/mL; indicating that ewes were not pregnant.

Recognition of placental or fetal structures as a criterion of pregnancy precludes the possibilities of making false positive diagnoses. However, prior to Day 45 of gestation, the fluid–filled vesicle is the most prominent sign of pregnancy and the fetal or the placental structures sometimes are missed during transrectal ultrasonographic examination [3]. This most likely explains the significantly higher specificity (fewer false positive diagnoses) of the transrectal ultrasonography at Day 50 than that of the test at Days 29 and 36 of gestation. The probable reasons for the false positive diagnoses are discussed in Experiment 2.

In Experiment 2, the overall sensitivity (92 %) of the transrectal ultrasonography for detecting pregnant ewes was higher than that (87 %) obtained in another study between Days 25 to 50 of gestation [5]. On the other hand the specificity of the test for detecting non-pregnant ewes in both studies were the same. In that study, ewes were fasted for 12 hours before the ultrasonographic examinations, but the abdominal wall of the animal was not lifted up during scanning as in our study. In our Experiment 2, 6 false negative diagnoses were made by transrectal ultrasonography at Day 24 of gestation. All of them had ovPAG concentrations higher than the threshold of pregnancy. At Days 20 to 25 of gestation, the amount of embryonic fluid is small, and therefore it is difficult to be detected by a 5 MHz linear-array transducer, especially in large and mature ewes [2-3]. With one exception, these false negative ultrasonographic diagnoses were confirmed as correct positive ones by examining the ewes at Days 29 and 34 of gestation. Therefore these results emphasize the importance of re-examination of ewes with negative transrectal ultrasonographic diagnoses performed before Day 29 of gestation.

Seven false positive diagnoses were made by transrectal ultrasonography at Day 24 of gestation; three of them were also incorrectly diagnosed as pregnant at Days 29 and 34 of gestation. All the seven ewes had ovPAG concentrations lower than the threshold level for pregnancy. This also confirms that these ewes were non-pregnant at the time of scanning. Metritis, pyometra and hydrometra or any unknown conditions associated with accumulation of anechoic intrauterine or abdominal fluid might be the reason of the false positive diagnoses made by transrectal ultrasonography in both experiments [29-31].

Regarding PAG-RIA test, all lambed or aborted ewes had ovPAG concentrations higher than the threshold level for pregnancy in both experiments. In addition, with three exceptions, all non-pregnant ewes had undetectable ovPAG levels or lower than the threshold level for pregnancy during the examined periods in both experiments. One ewe was diagnosed as non-pregnant by ultrasonography at Day 24 of gestation and had ovPAG level (2.3 ng/mL) higher than the threshold of pregnancy, but it was lower than the average of ovPAG concentration (5.8 ± 2.3 ng/mL) in that day. Thereafter, the ovPAG concentration decreased to 1.2 ng/mL at Day 29 and 1.1 ng/mL at Day 34, respectively. Embryonic mortality might have occurred in this ewe before d 24 of gestation. The other two ewes had ovPAG levels only just above the threshold level at d 29.

Similar to the results reported in our previous study [8], fewer false negative and positive diagnoses were made by transrectal ultrasonography in Experiment 2 than that made in Experiment 1. The probable reasons may be the effect of the fasting and/or lifting the abdomen of the animals performed in Experiment 2. Fasting ewes 12 to 24 hours prior to the scanning reduces the intestinal gases or ingesta, which might interfere with the identification of the pregnant uterus or induce image artifacts [3, 29]. In addition, lifting the abdomen of the ewes by the hand of the assistant may push the reproductive tract, especially in large breed to the pelvis to be within reach of the ultrasound beam.

Comparing with P4 test, PAG RIA test is more specific because it can differentiate between pregnancy and prolonged inter-estrus intervals [21]. In addition, unlike P4 test, timing of blood sampling for PAG-RIA test is not dependent on the knowledge of the exact estrus. The PAG-RIA test is more accurate than transrectal ultrasonography for early pregnancy diagnosis (Day 24). However, transrectal ultrasonography has the advantage over PAG-RIA of being on-farm test. Currently, efforts are being made to develop ELISA kits for PAG to enable the producer to apply the test on farm.

In conclusion, the accuracy of transrectal ultrasonography for detecting early pregnancy in sheep can be evaluated by measurement of plasma ovPAG concentrations. The heterologous PAG-RIA is more accurate than tranrectal ultrasonography for diagnosing pregnant and non-pregnant Awassi x Merino ewes at Day 24 of gestation. Furthermore, transrectal ultrasonography is an accurate method for pregnancy diagnosis after Day 24 of gestation when the Awassi x Merino ewes are fasted before scanning and their abdominal wall is lifted up during the scanning.

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Table 1

Sensitivity, specificity and predictive values of transrectal ultrasonography for early pregnancy diagnosis in Awassi x Merino ewes (Experiment 1).

Days of pregnancy	No. of ewes	a	b	c	d	Se %	Sp %	+ PV %	-PV %
29	156	16	21	104	15	51.6	83.2 [e]	43.2	87.4
36	155	13	14	110	18	41.9	88.7 [f]	48.1	85.9
50	148	15	3	116	14	51.7	97.4 [e f]	83.3	89.2

a: correct positive diagnosis (pregnant), b: incorrect positive diagnosis (non-pregnant), c: correct negative diagnosis (non-pregnant), d: incorrect negative diagnosis (pregnant), Se: sensitivity, Sp: specificity, +PV: positive predictive value, -PV: negative predictive value. Percentages within a column and with the same superscript are different ([e] P=0.0003; [f] P= 0.0127, respectively).

Table 2

Sensitivity, specificity, and predictive values of transrectal ultrasonography for early pregnancy diagnosis in Awassi x Merino ewes (Experiment 2).

Days of pregnancy	No. of ewe	a	b	c	d	Se %	Sp %	+ PV %	-PV %
24	268	26	7	229	6	81	97	78.7	97.4
29	268	31	3	233	1	96.8	98.7	91.1	99.5
34	251	31	3	216	1	96.8	98.5	91.1	99.5

a: correct positive diagnosis (pregnant), b: incorrect positive diagnosis (non-pregnant), c: correct negative diagnosis (non-pregnant), d: incorrect negative diagnosis (pregnant), Se: sensitivity, Sp: specificity, +PV: positive predictive value, -PV: negative predictive value.

Table 3

Age (Mean ± S.D.) of ewes with ultrasonographic false negative and correct positive diagnoses in different examination periods (Experiment 1)

Days of ultrasonographic examinations	Age of ewes (year)	
	Incorrect negative diagnoses	Correct positive diagnoses
29	5.3 ± 1.8 [a] (*n*=15)	3.6 ± 2.3 [b] (*n*=16)
36	5.3 ± 2.3[c] (*n*=18)	3.2 ± 1.5 [d] (*n*=13)
50	5.6 ± 1.74 [c] (*n*=14)	3.3 ± 2.1 [d] (*n*=15)

[a, b] $P< 0.05$.

[c, d] $P< 0.005$.

Table 4

Concentrations of ovine pregnancy-associated glycoprotein (ovPAG) in pregnant ewes incorrectly diagnosed as non-pregnant (false negative diagnoses) by transrectal ultrasonography (US) in Experiment 2

Ewe ID	Day 24		Day 29		Day 34	
	US	ovPAG (ng/mL)	US	ovPAG (ng/mL)	US	ovPAG (ng/mL)
A	NP	7.7	NP	10.3	P	5.5
B	NP	3.1	P	8.5	P	6.2
C	NP	5.2	P	13.5	P	7.8
D	NP	6.6	P	6.2	P	5.5
E[*]	NP	13.8	P	11.9	P	15.4
F	NP	4.9	P	6.8	P	7.0
G	P	8.1	P	16.8	NP	7.2

P: pregnant; NP: non-pregnant.

[*]This ewe was inseminated at the same day of the other ewes. By transrectal ultrasonography at Days 29, 34 and 50, her pregnancy appeared to be older compared with other pregnancies. This ewe aborted after Day 50 of gestation. It may be conceived earlier by natural mating.

Table 5

Concentrations of ovine pregnancy- associated glycoprotein (ovPAG) in non-pregnant ewes incorrectly diagnosed as pregnant (false positive diagnoses) by transrectal ultrasonography (US) in Experiment 2

Ewe ID	Day 24		Day 29		Day 34	
	US	ovPAG (ng/mL)	US	ovPAG (ng/mL)	US	ovPAG (ng/mL)
A	P	Undetectable	P	Undetectable	P	Undetectable
B	P	Undetectable	P	Undetectable	P	Undetectable
C	P	Undetectable	P	Undetectable	NP	0.3
D	P	Undetectable	NP	Undetectable	P	0.4
E	P	Undetectable	NP	Undetectable	NP	Undetectable
F	P	Undetectable	NP	0.4	NP	0.3
G	P	Undetectable	NP	0.5	NP	0.4

P: pregnant; NP: non-pregnant

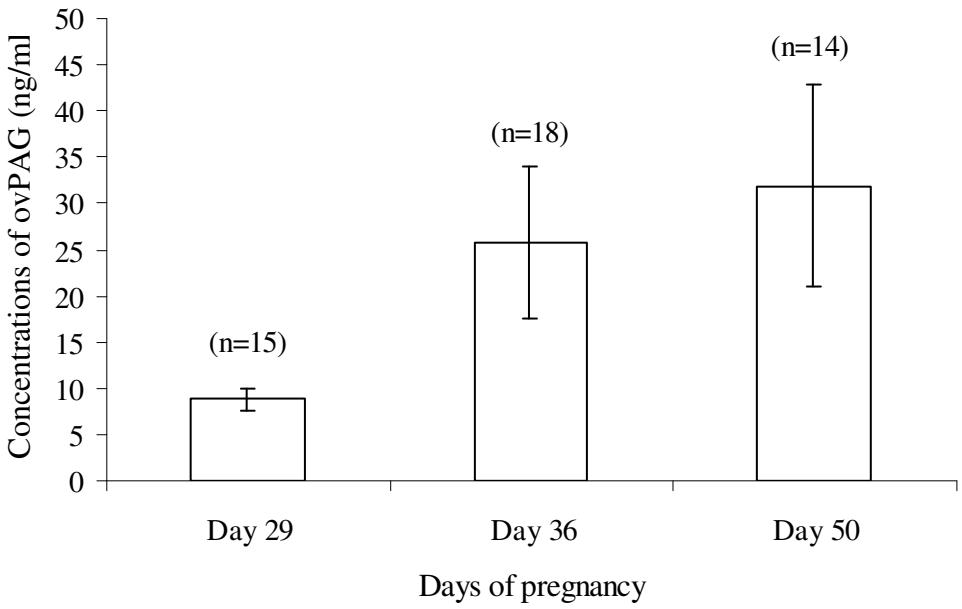

Figure 1. Mean (± S.D.) plasma concentrations of ovine pregnancy-associated glycoprotein (ovPAG) in ewes incorrectly diagnosed as non-pregnant (false negative diagnoses) by transrectal ultrasonography in Experiment 1.

CHAPTER 5

SUMMARY AND CONCLUSIONS OF THE THESIS

Accurate diagnosis of early pregnancy is a key factor for successful reproduction management in sheep farm. Among the numerous methods used for pregnancy diagnosis in sheep, a few methods can diagnosis early pregnancy. However, these methods are either impractical or not specific for pregnancy. Recently, pregnancy-associated glycoproteins (PAG) have been isolated from domestic ruminant placentas and radioimmunoassays have been developed for detecting PAG in the maternal blood or milk of pregnant animals. These radioimmunoassays have been used accurately for detecting early pregnancy in cattle and goats. However, there is no data about the accuracy of pregnancy associated glycoprotein-radioimmunoassay (PAG-RIA) for early pregnancy diagnosis in sheep. Transrectal ultrasonography has been recommended as a practical test for early pregnancy diagnosis in sheep. The variable accuracy of transrectal ultrasongraphy for early pregnancy diagnosis in sheep led us to study the factors, which could improve the accuracy of the technique and to investigate the false ultrasonographic pregnancy diagnoses.

Chapter 1: **Pregnancy diagnosis in sheep: Review of the most practical methods**

Various practical methods have been used for pregnancy diagnosis in sheep. Both pregnancy and fetal numbers are accurately diagnosed by using radiography after Day 70 of the gestation. Rectal-abdominal palpation technique detects pregnancy with an accuracy of 66 to 100% from Days 49 to 109 of gestation, however it has a low (17 to 57%) accuracy for determining multiple fetuses. Progesterone assays have a high sensitivity (88% to 100%) and a low specificity (60% to 72%) at Days 16 to 18. Estrone sulphate assay accurately detects pregnant ewe at Days 30 to 35. Ovine pregnancy specific protein B (ovPSPB) assay accurately (100%) detects pregnancy from Days 26 after breeding onwards. The accuracy of progesterone, estrone sulphate and ovPSPB assays for determining fetal numbers is relatively low. A-mode and Doppler ultrasonic techniques accurately detect pregnancy during the second half of gestation. Fetal numbers can not be determined by A-mode ultrasound, while the Doppler technique needs experience to achieve high accuracy. Transrectal B-mode, real time ultrasonography identifies the embryonic vesicles as early as Day 12 after mating, but the sensitivity of the technique for pregnancy is very low (12 %) earlier than 25 days after mating. Transabdominal B-mode ultrasonography achieved high accuracy for pregnancy diagnosis (94 % to 100 %) and the determination of fetal numbers (92 % to 99 %) at Days 29 to 106 of gestation. Real-time, B-mode ultrasonography appears to be the most practical and

accurate method for diagnosing pregnancy and determining fetal number in sheep.

Chapter 2: **Early pregnancy diagnosis in sheep by progesterone and pregnancy-associated glycoprotein tests**

This is the first study to evaluate the accuracy of the pregnancy-associated glycoprotein radioimmunoassay (PAG-RIA) test for detecting early pregnancy in sheep and to compare it with that of progesterone radioimmunoassay (P4-RIA) test.

One hundred and eighty-two Awassi x Merino ewes were synchronized for estrus by insertion of intravaginal sponges containing 30-mg flurogestone acetate for 14 days at the beginning of the breeding season. At the time of sponge removal, all ewes were administered 600 IU eCG im. All ewes were inseminated twice with fresh semen at 48 and 56 h after sponge removal. Day of insemination was considered as Day 0 for calculating the gestational age. Blood sample was collected from each ewe at Days 0, 18, 22, 29, 36 and 50 after AI.

Plasma concentrations of P4 at Days 0 and 18 after AI were measured by a double-antibody radioimmunoassay. Ovine PAG concentrations at Days 22, 29, 36 and 50 after AI were determined by a heterologous double-antibody radioimmunoassay using boPAG 67 KDa as tracer and standard and rabbit antiserum raised against a mixture of caPAG 55+59 KDa as the first antiserum. The discriminatory value for diagnosis of pregnancy by P4 and PAG-RIA tests was ≥ 1ng /mL.

Based on the lambing data and any observed sign like abortion, the accuracy for detecting pregnant (sensitivity) and non-pregnant ewes (specificity), and predictivity of both tests were calculated. The sensitivity of P4 test at Day 18 after AI was 100 %, while the specificity of the test was 95.6 %, because 7 non-pregnant ewes had P4 levels higher than 1 ng/mL (false positive diagnoses). Two of these ewes had high P4 level (>1 ng/mL) at Day 0. Regarding PAG-RIA test, the sensitivity at Day 22 was 93.5 %, because 2 pregnant ewes had ovPAG level lower than the threshold for pregnancy. At Days 29, 36 and 50 after AI, the sensitivity of the test reached 100 %. The specificity of the test was 100% at Days 22, 36 and 50, while it was 99.2% at Day 29, because one non-pregnant ewe had PAG level (1.02 ng/mL) slightly higher than the threshold of pregnancy. The specificity of PAG-RIA test at Days 22, 36 and 50 after AI was significantly ($P < 0.05$) higher than that of P4-RIA test. On the other hand there was no significant difference in the sensitivity of both tests for detecting pregnant ewes.

From this study, we can conclude that the heterologous PAG-RIA test is a reliable method for diagnosing pregnancy in sheep with high accuracy from day 22 of gestation. The advantage

of PAG over P4 test is that it can differentiate between pregnancy and prolonged inter-estrus intervals.

Chapter 3: **Accuracy of transrectal ultrasonography for determination of pregnancy in sheep: Effect of fasting and handling of the animals**

This field study was designed to investigate the effect of fasting ewes before scanning and lifting up their abdominal walls during scanning on the accuracy of transrectal ultrasonography for pregnancy diagnosis.

Four flocks (A, B, C and D) of Awassi x Merino ewes (*n*=1247), aged 0.7 to 10 years, were synchronized for estrus and were artificially inseminated. Two weeks later, fertile rams were kept with ewes of flocks A, B and C (*n*=949) to mate those returned to estrus, while ewes of flock D (*n*=298) were re-inseminated 17 days after the first insemination. A total of 1713 ultrasound scans were made in 1247 ewes, whereas ewes of flock A, B and C were scanned on four separate occasions after separating the diagnosed pregnant ones.

All ewes were scanned by B-mode real-time ultrasound scanner (Aloka SSD 500) equipped with a 5 MHz transducer. The probe of the scanner was modified by taping a plastic rod along its ventral part to control the manipulation of the transducer inside the rectum. The same operator who had trained for ultrasographic pregnancy diagnosis in small ruminants carried out all the transrectal ultrasonograhic examinations. The ewes were divided into two groups: Group I (n=949) was scanned in the standing position at the milking parlor. Group 2 (n=764) was scanned by the same operator using the same technique of the examination applied in Goup 1. However, the ewes were fasted 12 hours before scanning and their abdominal wall in front of udder were lifted up by the assistant's hands during scanning. Depending on the stage of pregnancy, the recognition of allantoic fluid, embryo proper, placentome or fetus was considered as a positive sign of pregnancy.

The gestational age at the time of scanning was calculated either by considering day of insemination as Day 0 (for ewes conceived by AI) or by subtracting the interval from scanning till lambing from 150 day (for ewes conceived by natural mating).

Based on the lambing performance of tested ewes, the sensitivity, the specificity, and the positive and negative predictive values of the transrectal ultrasonography in both groups were calculated.

The sensitivity of the transrectal ultrasonography for diagnosing pregnancy at Days 18 to 24, 25 to 30, 31 to 40 and 41 to 50 was 21.8 %, 32.3 %, 63.3 % and 50 % in Group 1 and 46 %, 92.5 %, 92.3 % and 96.8 % in Group 2, respectively. The sensitivity of the test in Group 2

was significantly higher than that of the test in Group 1 in all examination periods (P< 0.01 at Days 18 to 25 and *P*< 0.0001 at Days 25 to 30, 31 to 40 and 41 to 50). The specificity of the test for diagnosing non-pregnant ewes at Days 18 to 24, 25 to 30, 31 to 40 and 41 to 50 was 92.7 %, 92.0 %, 91.7 % and 94.7 % in Group 1 and 92.0 %, 94.2 %, 94.8 % and 83.3 % in Group 2, respectively. No significant differences in the specificity of the test were observed between two groups with the exception of Days 41 to 50, whereas specificity of the test in Group 1 was significantly (*P*< 0.01) higher than that of the test in Group 2.

The sensitivity of the test was higher in young ewes (0.7 to 2 years) than that of the test in old ones (> 2 to 10 year) in Group 1. Significant differences were observed at Days 18 to 24 (P< 0.01) and 41 to 50 (*P*< 0.05) of gestation. On the other hand, the sensitivity of the test in young and old ewes was not significantly different in Group 2. The specificity of the test was similar in young and old ewes in both groups. In Group 1, the sensitivity of the test at Days 31 to 41 and 41 to 50 was significantly (*P*<0.0001, *P*= 0.002, respectively) higher than that of the test at Days 18 to 24 of gestation. Furthermore, a significant (*P* = 0.003) higher sensitivity was made by the test at Days 31 to 40 than that at Days 25 to 30 of gestation. In Group 2, The sensitivity of the test at Days 25 to 30, 31 to 40 and 41 to 50 was significantly (P< 0.0001) higher than that of the test at Days 18 to 24 of gestation.

In conclusion, fasting Awassi x Merino ewes for 12 hours before scanning and lifting up their abdominal walls in front of udder by the assistant's hands greatly improve the accuracy of the transrectal ultrasonography for detecting pregnancy.

Chapter 4: **Evaluation of false transrectal ultrasonographic pregnancy diagnoses in sheep by measuring plasma level of pregnancy-associated glycoproteins**

The aim of the present study was to investigate to what extent pregnancy diagnoses made by transrectal ultrasonography could be confirmed by the measurement of plasma ovPAG concentrations on the same day.

A total of 424 Awassi x Merino ewes were synchronized for estrus during the second half of August 2000 (*n*=156) and 2001(*n*=268), respectively. All ewes were artificially inseminated and the day of insemination was considered as Day 0 for calculating the gestational age.

Transrectal ultrasonographic examinations were conducted in two experiments by using a real-time ultrasound scanner (Aloka SSD 500) equipped by a 5 MHz transducer. In experiment 1, 156 ewes were scanned at Days 29, 36 and 50 after AI in standing position at the milking parlor. Recognition of allantoic fluid at Days 29 and 36 of gestation was considered as a positive sign of pregnancy. At Day 50, the recognition of the fetus or

placentomes was used as a criterion for a positive pregnancy diagnosis. In experiment 2, 268 ewes were scanned by the same operator using the same technique applied in Experiment 1, but ewes were fasted for 12 hours before scanning and their abdominal walls were lifted up by assistant's hands while conducting the scanning. Blood sample was collected from each ewe after each ultrasonographic examination. Concentrations of plasma ovPAG were measured by the heterologous double-antibody radioimmunoassay. The discriminatory value for diagnosis of pregnancy by PAG-RIA test was ≥ 1ng /mL.

Based on the lambing performance or any observed sign like abortion, the results of transrectal ultrasonography and PAG-RIA test were arranged into: correct positive diagnoses (pregnant), incorrect positive diagnoses (false positive diagnosis), correct negative diagnosis (non-pregnant) and incorrect negative diagnosis (false negative diagnosis). From these values, the sensitivity, specificity and predictivity of transrectal ultrasonography were calculated.

In Experiment 1, 47 false negative diagnoses (15 at Day 29, 18 at Day 36 and 14 at Day 50 after AI, respectively) were made in 24 ewes by transrectal ultrasonography. In addition, 38 false positive diagnoses (21 at Day 28, 14 at Day 36 and 3 at Day 50 after AI, respectively) were made in 33 ewes by the test. When the recognition of the fetus(es) or placentomes were used as a sign for positive pregnancy diagnoses at Day 50, a significant higher specificity was obtained compared with that of the test at Days 29 (P= 0.003) and 36 (P= 0.0127) after AI.

In experiment 2, 8 false negative diagnoses (6 at Day 24, 1 at Day 29 and 1 at Day 34, respectively) were made in 7 ewes by the transrectal ultrasonography. With one exception (at Day 29), all the false negative diagnoses made by the test at Day 24 were correctly diagnosed as pregnant at Days 29 and 34. Thirteen false positive diagnoses (7 at Day 24, 3 at Day 29 and 3 at Day 34 after AI, respectively) were made in 7 ewes by the test. In both experiments, all ewes with false negative diagnoses had ovPAG levels higher than the threshold for pregnancy (≥ 1ng /mL). Interestingly, all ewes with false positive diagnoses had ovPAG level lower than the threshold of pregnancy, therefore theses ewes were not pregnant at the time of transrectal ultrasonography. Regarding PAG-RIA test, all pregnant ewes had PAG level higher than the threshold for pregnancy during the examination periods in both experiments. With three exceptions, all non-pregnant ewes had ovPAG levels lower than the threshold of pregnancy. These three ewes were diagnosed as non-pregnant by transrectal ultrasonography during the examined days. One of the three exceptional ewes suffered from embryonic mortality before Day 24 after AI. In conclusion, PAG-RIA test greatly confirms and

interprets false transrectal ultrasonographic pregnancy diagnoses in Awassi x Merino ewes from Day 24 after artificial insemination.

CONCLUSIONS

The pregnancy rate obtained in the present thesis after artificial insemination of ewes synchronized for estrus at the beginning of the breeding season was low. Similar results were obtained following artificial insemination or natural mating of the ewes in synchronized estrus during or at the end of the breeding season (Acritopoulou-Fourcroy et al., 1982; Garcia et al., 1993).

Results of the present thesis show that radioimmunoassay of the plasma progesterone at Day 18 after AI detects all pregnant ewes. However the specificity of the test for detecting non-pregnant ewes is relatively low, because plasma level of progesterone ≥ 1 ng /mL indicates a functional corpus luteum which associates with pregnancy, pathological cases such as pyometra and hydrometra, luteal cysts, embryonic mortality or irregular estrus cycles (Mukasa-Mugerwa and Viviani, 1992, Ishawer, 1995). The heterologous double-antibody radioimmunoassay using boPAG as standard and tracer and the antiserum raised against caPAG 55+59 as the first antibody accurately determines pregnant and non-pregnant ewes from Day 22 after AI. Because PAG are secreted from placenta therefore, its presence in maternal plasma in a level higher than the threshold indicates the presence of pregnancy. Therefore, PAG-RIA test is more specific than progesterone test for detecting non-pregnant ewes and unlike progesterone test, can differentiate between pregnancy and prolonged inter-estrus interval. Our results indicates that PAG-RIA test is more accurate than transrectal ultrasonography (5 MHz) for detecting pregnant ewes at Day 24 of after AI. When the day of insemination is known, a single blood sample is required for PAG-RIA test to accurately differentiate between pregnant and non-pregnant ewes from Day 22 after AI. The potential using the PAG-RIA test in a modified kit form will enable the sheep producer to apply the test in the farm and over come the time delay and the hazard of radiation.

From the results of the thesis, we can also conclude that 5 MHz transrectal ultrasonography is an accurate and practical method for pregnancy diagnosis in Awassi x Merino ewes from Day 29 After AI and when ewes are fasted for 12 hours before scanning and their abdominal wall is lifted up while conducting scanning. In addition, using the embryo proper, fetus or placentome with allantoic fluid as a positive sign of pregnancy is recommended to avoid the false positive diagnoses made by transrectal ultrasonography.

Using of B-mode real time ultrasonography for simple pregnancy diagnosis in sheep would be economical to farm management when the pregnancy rate is lower than 90 % (Sprecher et al., 1989).

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NEW FINDINGS OF THE THESIS

- The heterologous radioimmunoassay for pregnancy associated glycoproteins (PAG) is a reliable and accurate method for determining pregnant and no-pregnant ewes from Day 22 after breeding.

- Fasting Awassi x Merino ewes for 12 hours prior to scanning and lifting up the abdominal wall of the animal during scanning greatly improve the accuracy of transrectal ultrasonography for diagnosing pregnancy.

- Transrectal ultrasonography with a 5 MHz transducer is an accurate technique for pregnancy diagnosis in Awassi x Merino ewes from Day 29 of gestation.

- PAG-RIA test is more accurate than transrectal ultrasonography for diagnosing early pregnancy in Awassi x Merino ewes. In addition the test is more specific than progesterone test for detecting non-pregnant ewes.

ACKNOWLEDMENTS

I am greatly indebted to:

Professor Ottó Szenci (Supervisor of this work and Head of Clinic for Large Animals, Faculty of Veterinary Science, Budapest) for his wise and instructive supervision and for his excellent international cooperation which allowed me to make my investigations within the international standards. Without his initiative and constructive advice, this study would not have been possible. I am also thankful for his patience and kindness.

Professor Jean-Francios Beckers (Co-promotor of this work and Head of Department of Physiology of Reproduction, Faculty of Veterinary Medicine, Liége, Belgium) for his unlimited support and continuos encouragement. Special thanks for his constructive scientific comments and advice.

Dr. Péter Kovács (Director of the Awassi Corporation, Bakonszeg) for his excellent cooperation and support. I would like to thank him for providing the facilities and animals to carry out the experiments of this work.

Dr. Jose Sulon (Department of Physiology of Reproduction, Faculty of Veterinary Medicine, Liege, Belgium) for his great help in the laboratory part of this work.

Dr. Reiczigel Jenő (Department of Biomathematics and Informatics**,** Faculty of Veterinary Science, Budapest) for his help in the statistical analyses.

Professor M.A.M Taverne (Utrecht University, The Netherlands) for his valuable comments

Professor Làszló Solti (Dean of Faculty of Veterinary Science, Budapest) and **Professor Gyula Huszenicza** (Deputy Dean for Postgraduate Studies) for their sincere efforts to provide the excellent opportunity to do my Ph.D. work at the faculty of Veterinary science in Budapest.

Mr. **Szabados Krisztiàn** for his help during carrying out the experiments in the farm.

Hungarian Ministry of Education for granting a 3-year scholarship which enable me to accomplish this work.

Egyptian Ministry of Higher Education represented in **Egyptian Cultural Bureau, Budapest**, for providing the financial support during the period of the study.

All the staff members of the **Department and Clinic of Obstetrics and Reproduction**, Budapest, **Department of Physiology of Reproduction**, Liege, Belgium and **Awassi Corporation**, Bakonszeg, are acknowledged for their supporting milieu.

my parents for their kindness, encourgement and patience. And finally my wife for her help in blood sampling, her love and for providing a stable family backgrounds which make me steady in these years.

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