Indian Journal of Animal Sciences **82** (11): 1317–1322, November 2012

Effect of different treatments on ovarian condition and relative variation of steroids and insulin-like growth factor I in serum during induction of oestrus of seasonally acyclic sheep

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Received: 19 September 2011, Accepted: 4 May 2012

ABSTRACT

Hormonal treatment during the non-breeding season is effective at precise synchronization of oestrus for fixed time insemination with the ability to increase ovulation rate in ewes. Three methods of assisted oestrus in Vallachian ewes were studied during the non-breeding season – group A: control, groups B, C: application of 20 mg flugestone acetate impregnated intravaginal sponges for 12 days + 1000 IU/500 IU equine chorionic gonadotrophin, respectively, on day of sponge withdrawal, group D/ovsynch protocol (day –9: lecirelinum 0.0125 mg/head, day –3: cloprostenolum 0.0375 mg/head, day -1: lecirelinum 0.0125 mg/head), n=6 for each. Laparotomy was carried out on ewes of all groups 24 h after the last treatment. Ovaries were collected, measured, and their volume was calculated, surface follicles were counted and measured, too. Blood was collected on the last day of treatment and on the day of laparotomy and progesterone (P_4), oestradiol-17ß (E_2), and insulin-like growth factor I (IGF-I) concentrations were analyzed in blood serums. E_2 concentrations were significantly higher on day of laparotomy in groups A and C. P_4 and IGF-I concentrations did not vary between or among groups. Volumes of the ovaries were significantly higher in control group when compared to groups B and D. Not a single treatment affected number or size of the follicles on the ovaries of Vallachian sheep positively, regardless the most effective treatment for assisted oestrous in the non-breeding season is found to be the use of progestogen vaginal sponges with 1000 IU eCG which may increase the ovulation rate.

Key words: Anoestrous sheep, IGF-I, Intravaginal sponge, Oestradiol-17 beta, Ovsynch, Progesterone

Assisted oestrus might be helpful in augmentation of fertility and production in sheep, a known seasonal breeder, by utilization of different methods of hormonal or non-hormonal treatment (Knights *et al.* 2001, Zarkawi *et al.* 2001, Maracek *et al.* 2002, Maracek *et al.* 2004, Vlèková *et al.* 2005). Hormonal treatment is effective but requires expense for pharmacological preparations and their application. Non-hormonal treatment is cheaper but does not guarantee the precise oestrus synchronization and is limited by certain/specific conditions such as breeding conditions and season (Evans and Maxwell 1987, Haresign 1992, Henderson and Robinson 2000). One of the most commonly used hormonal treatments is application of progesterone or its synthetic analogues (Cognie 1990). Application of progestogens may shorten the lifespan of corpus luteum, hence, in case of effective synchronization the duration of its action must reflect a luteal phase of the oestrus cycle (Godfrey *et al.* 1999, Bari *et al.* 2001, Maracek *et al.* 2004). In anoestrous ewes, progestogen must be replaced by follicle stimulating preparations (e.g. equine chorionic gonadotropin, eCG) to induce growth of follicles, oestrus, and ovulation (Haresign 1992, Godfrey *et al.* 1999, Bari *et al.* 2001). This gonadotropin should be applied in higher doses in anoestrous ewes than that in cycling ewes (Cognie 1990, Maracek *et al.* 2002, Maracek *et al.* 2004).

Use of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its analogues for oestrus synchronization terminates the luteal phase of the oestrous cycle in cycling ewes by a regression of corpus luteum resulting in decrease in progesterone in blood (Ishwar and Memon 1996). For optimal synchronization 2 injections are required (Wildeus 1999) during days 10 to 14 of the oestrous cycle (Ishwar and Memon 1996, Wildeus 1999). Oestrus will appear in 2 to 3 days, but without enough precision for fixed time insemination (Mutiga and Mukasa-Mugerwa 1992). Some authors have reported the use of $PGF_{2\alpha}$ with GnRH in cycling ewes (Taponen *et al.* 2002, Cárdenas *et al.* 2004, Deligianis *et al.* 2005). Their results

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indicated that use of modified ovsynch protocol in cycling ewes can achieve acceptable conception rate, which would be further improved by modifying the intervals between injections. Use of modified ovsynch protocol (Deligianis *et al.* 2005) indicated lower fertility and fecundity (Vlcková *et al.* 2008, Vlcková *et al.* 2010) when compared to the ewes treated with progestogen and 500 IU eCG, but with the same percentage of prolificacy. Effect of this treatment on the reproductive system for assisted oestrus during the non-breeding season has not yet been studied. It was only reported that gonadotropins with FSH activity and GnRH (LHRH) induce the ovulation in anoestrous ewes when used alone (Evans and Maxwell 1987, Cognie 1990, Maracek *et al.* 2002, Maracek *et al.* 2004). In anoestrous ewes, folliculogenesis and gonadotropin-sensitive follicles and their selection occurs in periodic growth waves with the same quality of dominant follicles and their oestrogenic activity as during the breeding season, except for a terminal phase of maturation due to deficient release of GnRH and LH to provoke ovulation (Evans *et al.* 2000, Notter *et al.* 2003, Laurincik and Strejcek 2005). However, Vlèková *et al.* (2008b, 2012) referred the occurrence of corpora lutea in the first month after spring parturition. The aim of the study was to compare various treatments of assisted oestrus of ewes during the non-breeding season to verify the effects of these treatments on the ovarian hormonal (progesterone, oestradiol-17beta), insulin-like growth factor I (IGF-I) serum concentrations, and foliculogenesis in the time of expecting ovulation and to observe potential ovulation rates on the ovaries. The other aim was to confirm our previous theory that the fully running folliculogenesis and luteogenesis occur in anoestrous ewes similarly as in postpartum ewes.

MATERIALS AND METHODS

Seasonal acyclic Improved Vallachian breed ewes (24), 4– to 6- year-old, weighing 45 to 50 kg, were divided into 4 groups each group comprising 6 animals. The experimental groups were control (A), B, C, and D. The ewes of groups B and C were treated with intravaginal sponges containing 20 mg FGA (flugestone acetate, cronolone) and kept *in situ* for 12 days. On the day of sponge withdrawal an intra-muscular injection of eCG (equine chorionic gonadotropin) was given @ 1000 and 500 IU, respectively (Day -1). GnRH analogue lecirelinum was administered @ 0.0125 mg to each ewe of the group D (ovsynch) and after 5 days of this injection the analogue of $PGF_{2\alpha}$ - cloprostenolum @ 0.0375 mg was injected to each ewe and followed by a second dose of GnRH analogue 48 h of prostaglandin (Day -1). Experimental ewes were fed trefoil-grass silage, oat grain 0.5 kg/head/day, and hay and water *ad lib*. Jugular blood samples were collected aseptically, serum was separated and stored at –20°C after centrifugation for 15 min at 3000 rpm and late on used for estimation of concentrations of progesterone and oestradiol-17 beta using radioimmunoassay (RIA) and insulin-like growth factor I (IGF-I) using enzyme-linked immunosorbent assay (ELISA). Samples of the blood serum for steroids were assessed in duplicates. Analytical sensitivity (limit of detection) of progesterone was 0.03 ng/ml and the intra-assay and inter-assay coefficients of variation were ≤5.4% and ≤ 9.1%, respectively. Analytical sensitivity for oestradiol-17 beta was 4 pg/ml and the intra-assay and inter-assay coefficients of variation were ≤15.1% and ≤14.4%, respectively. Blood serum samples for IGF-I analyses were assessed in triplicate. Absorbance was measured in micro plate by ELISA reader at wavelength 450 nm. After starvation for 12 to 18 h, laparotomy was done (Day 0) under general anaesthesia with sodium pentobarbital as per Hall *et al.* (1993). After exploration the ovaries were removed carefully to study different parameters. Volumes of the ovaries were measured and expressed in cm^3. All antral follicles visible on the ovary surface were counted and classified into 3 categories. Follicles with the diameter equal or larger than 1 mm (F ≥1mm), follicles with the diameter equal or larger than 3 mm (F≥3mm), and follicles with the diameter equal or larger than 5 mm (F≥5mm). Their maximum diameter was estimated from the mean of 2 measurements of the follicle taken at right angles to each other. Follicles < 1mm in diameter were not included. Presence of corpus luteum was studied, too. The sizes of the ovaries, numbers of follicles in all 3 categories of diameters and hormone concentrations were statistically analyzed by arithmetic mean and its SE. Controls were compared with treated groups by one-way ANOVA with Tukey's and Dunn's post tests. Statistical significance was marked with superscript stars and defined as $P<0.05$ and $P<0.01$.

RESULTS AND DISCUSSION

The mean concentrations of serum progesterone did vary significantly between groups and days (Table 1). No corpora lutea were found on the ovaries of all groups, however groups A, B, and C showed relatively high progesterone levels. In control ewes (group A), the concentrations of progesterone were observed on the level higher than 2 ng/ml which may reflect the preovulatory LH surge when the concentrations of progesterone in the follicular fluid increase and begins to be dominant (Hanukoglu 1992, Bartlewski *et al.* 2000, Logan *et al.* 2002) and accompanied with a luteinisation of the follicle tissue (de Loos *et al.* 1991), while the concentrations of oestradiol-17 beta decreased to the minimal values (Campbell *et al.* 1990). This hormonal status may indicate the running folliculogenesis during anoestrus.

The concentrations of serum progesterone in group C were higher (>1 ng/ml) on day of laparotomy (Day 0) compared to the day before (Day -1, injection of 500 IU eCG) and this observation is in an agreement with the study of Iida *et al.* (2004) who reported that the concentrations of progesterone in anoestrous ewes at sponge withdrawal are generally around 1 ng/ml and 24 h later below this level, but it is not true for

Table 1. Mean±SE of progesterone, oestradiol-17 beta and IGF-I concentrations in anoestrous ewes

	Progesterone (ng/ml)		Oestradiol-17 beta (pg/ml)		IGF-I (ng/ml)	
Ewes	Day -1	Day 0	Day -1	Day 0	Day -1	Day 0
A	2.49±0.91	2.77±1.12	15.03±1.83	4.80±1.13**	312.90±71.05	349.40±62.45
B	0.96±0.32	1.79±1.15	44.01±8.39	86.36±22.80	318.30±43.25	243.80±12.99
C	1.73±0.15	0.92±0.27	58.41±14.86	92.06±18.33**	339,00±38.86	301.90±31.19
D	0.29±0.06	0.31±0.06	93.18±24.34	77.21±22.17	472.50±48.03	387.30±54.59

**P<0.01, A, control;B, FGA+1000 UI eCG; C,FGA+500 UI eCG;D, ovsynch protocol; Day -1 – day of sponge withdrawal and eCG injection (groups B and C) and/or the second injection of lecirelinum (group D), Day 0 – day of ovariectomy 24 h after the last treatment.

group B in the present study where the situation was *vice versa*. It could be presumed that the superovulatory dose of eCG (1000 IU in the present study) resulted in higher follicle luteinisation (de Loos *et al.* 1991) similarly as in control ewes.

The mean serum concentrations of oestradiol-17 beta (Table 1) in control group was very low in comparison with group C (P<0.01) where the oestradiol secretion might be resulted from binding of LH on its receptors in granulose cells to stimulate the synthesis of androgens and from FSH inducing the aromatization of this substrate to oestradiol in granulose cells (Perks *et al.* 1995, Webb *et al.* 2004). It is generally known that the secretion of androstendione and oestradiol is significantly higher from large follicles than from small antral follicles (Scaramuzzi *et al.* 1993, Gougeon 1996, Souza *et al.* 1998, Bartlewski *et al.* 1999, Evans *et al.* 2000). We observed that the concentrations of oestradiol-17 beta increased from Day -1 to Day 0 in groups B and C. This increasing secretion of oestradiol during follicular phase of the oestrous cycle may reflect high level of preovulatory follicle maturation associated with the increase in the number of LH receptors in both granulose and theca cells (Campbell *et al.* 1990).

Increased plasma concentrations of oestradiol in combination with low progesterone concentrations during follicular phase relates with a high frequency and low amplitude of LH secretion (Schrick *et al.* 1993). This increase in LH pulsatility protects the dominant follicle to undergo atresia and stimulates its development to the preovulatory stage (Savio *et al.* 1993). Treatment with eCG increases serum concentrations of oestradiol during the periovulatory period mainly in anoestrous ewes where this probably results in synchronous oestrus and ovulation (Barret *et al.* 2004).

Low levels of progesterone in serum in group D might have effect on follicular growth thereby increased serum oestradiol-17 beta. PGF_{2alpha} injection 7 days after the first GnRH injection in group D causes regression of CL or luteinized follicles and 48 h later, application of a second dose of GnRH should provoke LH release and ovulation of a dominant follicle (Martinez-Tinajero *et al.* 2011).

Concentrations of IGF-I (Table 1) did not show significant variances among or within groups, though in treated groups they decreased slightly from Day -1 to Day 0. Concentrations in group B conversely increased and this may indicate higher luteinisation of the follicles (Armstrong *et al.* 2001). IGF-I stimulates proliferation of granulose cells in small antral follicles and steroidogenesis by these cells of large antral follicles, where it is present in the follicular fluid in high concentrations (Monget *et al.* 2002). The present study showed that IGF-I concentrations were higher in ewes of group D and it could be presumed that this factor stimulates granulose cells proliferation and steroidogenesis and inhibits apoptosis (Armstrong *et al.* 2001, Sirotkin *et al.* 2003, Webb and Campbell 2007, Silva *et al.* 2009).

The right sided and the left sided ovaries were evaluated together, because they were similar in observed morphometrical values within individual groups. The lengths of the ovaries were nearly 2 cm in all groups. Ovaries of control group were longer (P<0.05) than those of group B (2.17±0.10 cm vs 1.75±0.07 cm), which is not in an agreement with a study of Maraèek (1992), where left ovaries dominated after use of progestogen and eCG treatment used in the same sheep breed. Mean volume of the ovaries (Fig. 1) in control was greater than in groups B and D (P<0.05, 3.73±0.62 cm^3 vs 1.74±0.3 cm^3 and 1.95±0.2 cm^3, respectively).

Total number of surface antral follicles was higher on the ovaries of ewes of group C in comparison with group D (P<0.05, 4.23±0.69 vs 1.96±0.32). No significant effect of any hormonal treatment on the number of follicles with

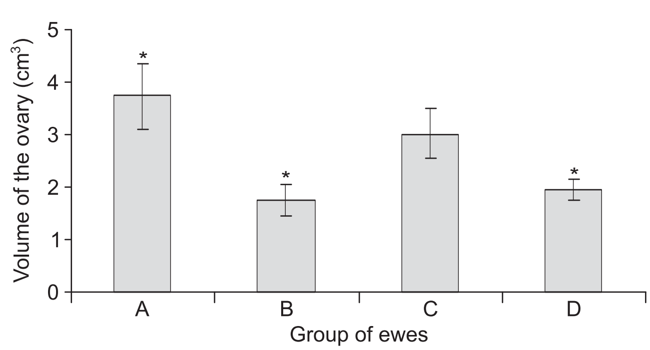

Fig. 1. Mean±SE. Volumes of the ovaries after FGA+1000 UI eCG (B), FGA+500 UI eCG (C), ovsynch protocol (D) treatment and control (A) of ewes on day of laparotomy.

Table 2. Numbers of follicles on the ovarian surface on day of laparotomy (Day 0)

Follicle number	A	B	C	D
F≥5mm	1.58±0.23	1.25±0.41	0.75±0.25	0.75±0.21
F≥3mm	4.58±1.04	4.33±1.04	3.17±1.02	1.92±0.62
F≥1mm	4.75±1.18	5.00±1.37	2.58±0.56	1.92±0.50

(n=12); A, control; B, FGA+1000 UI eCG; C, FGA+500 UI eCG; D, ovsynch protocol.

diameter ≥5mm and ≥3mm (Table 2) was observed. The highest number of these types of follicles was found in control group which are similar as the numbers of follicles observed previously in postpartum ewes (Vlcková *et al.* 2008b). The highest number of follicles F≥1mm was observed in group B. Mean follicle diameter was nearly equal in all studied groups (A,B,C,D, 0.36±0.04 cm, 0.32±0.02 cm, 0.30±0.02 cm, 0.28±0.01 cm, respectively) and showed no significant difference.

The largest follicle diameter was observed in group C (1.10 cm). In control group the diameter of the largest follicle was 1.00 cm and in groups B and D 0.99 cm; these correlate with used sheep breed as it is characterized to be monovulatory. Several authors described preovulatory follicles in sheep with monovulations as follicles larger than 5 mm (Bartlewski *et al.* 1999, Evans *et al.* 2000, Duggavathi *et al.* 2001). In the present study, the number of follicles ≥ 5mm was lower in group C than in group B. Synthetic progestogens and higher doses of eCG support the selection in the recruited follicle cohort resulting in higher number of dominant follicles that are able to ovulate and increased the ovulation rate (Maracek 1991). It could be presumed that 1000 IU eCG resulted into higher occurrence of atresia than lower doses (which correlated with the lowest levels of IGF-I in group B) as observed by Vlèková *et al.* (2008b). Sheep follicles with ≥3 mm diameter are the main source of oestradiol-17 beta (Souza *et al.* 1998, Evans *et al.* 2000). Our results presumed that using higher doses of eCG during the seasonal anoestrus of ewes resulted in higher number of oestradiol-producing follicles and more preovulatory follicles, which are able to produce high amount of progesterone leading to earlier ovulation, and the secretion of preovulatory secretion of progesterone is stimulated also by IGF-I in granulose cells (Bartlewski *et al.* 2000, Logan *et al.* 2002).

Our results showed that the treatment with progestogen and 1000 IU eCG is the most effective for the assisted oestrus during the non-breeding season, however, it may result in follicular cystic atresia in some animals as was observed previously (Vlcková *et al.* 2008a).

It could be concluded that both the growth and development of the ovarian follicles occur in the first third of non-breeding season with the presence of the preovulatory stage. Not a single treatment used in this study affected any observed parameter of the ovaries (volume of the ovaries and number of follicles) of improved Vallachian sheep positively, but the most effective treatment for the assisted oestrus during the non-breeding season is the treatment with progestogen and 1000 IU eCG which may also increase the ovulation rate.

ACKNOWLEDGEMENTS

Authors thank RN Dr Jana Kaatová for the assistance at sample collections and IGF-I assessment and RIA laboratory Košice, SR for analyses of progesterone and estradiol-17ß. This work was supported by the project of applied research AV 4/0113/06 and the grant of Ministry of education, science, research, and sport of the Slovak Republic VEGA 1/0860/09.

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