

THE USE OF THE OVINE PLACENTAL EXTRACT TO PREPARE ANESTRUS EWES TO INSEMINATION CAMPAIGN

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Abstract. In Ascanian Merino sheep the efficiency of animal treatment with ovine placenta tissue extracts only or complemented with substance of estrogenic ("Follikulin" 0.1%, 1 ml/an./inj.) or anticholinesterasal ("Prozerin" 0,05%, 1 ml/an./inj.) activities at anestrus period (July) as a way to prepare ewes to insemination campaign was examined. The placental extract was produced by method of academician Filatov and injected subcutaneously twice at interval of 5 days. Treatment with placental extract increased proportion of animals showed heat (E%) and fertility (F%), not affected fecundity (Fm%) and decreased prolificacy (Pf) against control. Additional injections of estrogenic substances changed few E%, improved F% and Fm%, increased Pf against control. Additional injection of anticholinesterasal agents not changed the impact of placenta extract. Treatment with placental extract only or complemented with other substances significantly increased index of heat for all research groups and decreased indexes of heat time and conception time, that indicates about improvement of results of insemination of treated animal during the first heat. Positive effect of treatment with placental extracts only or complemented with other substances was appeared in the reduction of proportion of animals with both the shortest (2–6 days) and extended (> 20) estrous cycles.

Keywords: ewe, placental extract, anestrus, insemination.

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1. Introduction

The preparing for insemination is responsible stage in the sheep breeding technology. The complex of traditional preparatory actions includes the increasing and improving of feeding level, treatment with antiparasitic drugs, the culling of animals. The preparing starts 1–1.5 months before the mating campaign that in sheep characterized seasonality of sexual activity is coincided in time with the end of summer or early autumn. With transfer of sheep breeding to an intensive way no less important gets the quality of renewal of sexual activity, in particular the timing and grouping of heat, ability to conceive at first insemination. A factor which is able to significantly affect the renewal of sexual activity in animals is hidden inflammation in the genital organs, because it is known that chronic endometritis reduces the LH peak amplitude [Peter et al., 1989] and impairs the development of follicles [Sheldon et al., 2002]. Usually these disorders are a sequela of complications of early postpartum period that became hidden during suckling. As a result of the fact that in sheep chronic inflammation of genitals almost do not show external signs and is not treated a certain percentage of

animals during insemination campaign neither shows sexual activity nor conceives. Therefore, we suggested that in addition to traditional the preparatory actions should include the measures to eliminate hidden disorders and stimulate sexual activity, and must be used not just before the beginning of the insemination campaign but in earlier anestrus season too.

One of ways for correction of hypofunctional status and treatment of chronic inflammation is placental extract, which get widespread use in cattle [Nikitina, Kacharyan, 2013]. Usually this preparation was made from the human or pig placenta. As for the extracts from ovine placenta, despite the large number of evidence of commercial use of it as part of rejuvenating creams and healing ointments, the number of researches of their influence on sheep is limited. It is worth mentioning the study of effect of ovine placental extracts on lactogen synthesis [Thordarson et al., 1987], development of udders and increase of ewe milk production [Cotor et al., 2011], strengthening of influence of biologically active substances [Mitchell et al., 1984], blocking the activity of certain enzymes [Al-Gabory et al., 1995]. Data on the use of ovine placental extract to correct reproductive status of ewes in particular anestrus season has not been found. Meanwhile, we had showed that double treatment of ewes in July with the placental extract increased proportion of animals that during the autumnal insemination campaign showed signs of heat and then lambled [Lobachova, Zhulinska, 2014].

The purpose of this research was to study the efficiency of using of ovine placental extracts in combination with substance of the estrogenic or anticholinesterasal activity as a way to prepare anestrus ewes to insemination campaign. The reason for use of these substances is dominant paradigm about neuro-hormonal mechanism of regulation of sexual activity in mammals. As a neurotropic it was chosen "Prozerin" characterized by anticholinesterasal activity and as an oestrogenic "Follikulin" contained natural hormone estrone.

2. Materials and methods

2.1 Place of research

The research was conducted in flock owned the Research Farm "Askania Nova" located at 46°27' north latitude. In Farm the breeder records registration had been introduced. Insemination campaign is usually carried out in two steps: at first after detection of heat presence animals are inseminated artificially with sperm of rams tested for sperm quality – the period of insemination, at second rams are added to ewes for free mating – the period of "add-mating".

2.2 Animals

In experiments 1.5–8-year old Ascanian Merino ewes were used. These sheep belong to breeds that are characterized by seasonal type of reproduction with the beginning to show the sexual activity at the third decade of August–the first decade of September and disappearance of ovulations in February–March. During experiment ewes and rams were kept apart in pens remote at a distance of at least

50 meters from each other. Contacts between males and females outside the insemination campaign were not allowed to prevent the display of "male effect". At beginning of experiment 4 groups were formed – 3 experimental ("PE", "PE+F", "PE+Pr") with 25 animals in each and 1 control ("C") with the rest animals of flock. All the ewes during time of observation were kept together with the same feeding level and free access to water. In summer and autumn months in morning hours animals free grazed. Prior to the insemination campaign 10 animals leaved research groups for independent reasons.

2.3 Manipulations with animals

The processing was begun July 3 by subcutaneous injections of placental extract (PE) to animals of all the experimental groups. The "PE+F" group additionally obtained subcutaneously "Folliculin" contained 0.1% estrone and the "PE+Pr" group – "Prozerin" contained 0.05% neostigmin methylsulphate. In September and October in the morning hours all the ewes were tested with teasers for heat and in case of detection were artificially inseminated with fresh non-diluted sperm. 4 days after end of period of heat detection ("insemination period") ewes were connected with rams for free mating ("add-mating period"). In February and March data about date and number of born lambs were recorded. The scheme of manipulations with ewes and doses of preparations used are presented in Tab. 1.

Table 1. Scheme of manipulations with ewes

Time	Groups			
	«TE»	«TE+F»	«TP+Pr»	«C»
03 July	PE, 4 ml, subc.	PE, 4 ml, subc. "Follicilin", 0.1%, 0.5 ml, subc.	PE, 4 ml, subc. "Prozerin", 0.05%, 0.5 ml, subc.	
08 July	PE, 3 ml, subc.	PE, 3 ml, subc. "Follicilin", 0.1%, 1 ml, subc.	PE, 3 ml, subc. "Prozerin", 0.05%, 1 ml, subc.	
09 September– 16 October	heat detection and insemination	heat detection and insemination	heat detection and insemination	heat detection and insemination
20 October – 10 November	free mating with rams	free mating with rams	free mating with rams	free mating with rams
03 February–31 March	date lambing and lamb number registration	date lambing and lamb number registration	date lambing and lamb number registration	date lambing and lamb number registration

2.4 Estimation of efficiency

Efficiency of the processing was evaluated with reproductive traits that are traditionally used for ewe groups – E%, F%, Pf and Fm%, and indexes of (seasonal) renewal of sexual activity that we developed for individual animals – IH_i , ITH_i , ICT_i and IL_i . Thus:

– E% – *index of sheep in oestrus* – estimated as quantity of ewes showed heat in insemination period divided by total number of ewes and multiplied by 100;

- F% – *fertility* – estimated as quantity of ewes lambing divided by total number of female and multiplied by 100;
- Pf – *prolificacy (or little size at birth)* – estimated as total number of lambs received divided by quantity of ewes lambing;
- Fm% – *fecundity* – estimated as quantity of all the lambs received divided by total number of ewes and multiplied by 100;
- IH_i – *index of heat* – equated to 1, if ewe showed heat during insemination campaign or lambing after "add-mating", and IH_i=0, if both the animal did not show heat and did not lamb after "add-mating";
- IHT_i – *index of heat time* – equated to 1, if ewe showed the first heat in the first 20 days of insemination campaign; IHT_i=2, if ewe showed the first heat after 20 days from beginning of insemination campaign; IHT_i=3, if ewe had not shown heat during insemination period but lambing after "add-mating"; IHT_i=4, if ewe had not shown heat during insemination period and did not lamb after "add-mating";
- ICT_i – *index of conception time* – equated to 1, if ewe had fertilized in the first shown heat, ICT_i=2, in the second heat, ICT_i=3, if animal had fertilized in the third or fourth heat, ICT_i=4, if ewe remained barren;
- IL_i – *index of lambing* – equated to 1, if ewe lambing single, 2 – twins 3 – triplets or more lambs, 0 – did not lamb.

As the reproductive traits of ewes are age-dependent to prevent the inadequate estimation because of predominance in group of animals of a certain age, indexes for each group were divided into 8 subgroups according to ewe age – 1, 2, 3 ... 7 and ≥8 full years. Final indexes for each group were determined by averaging of indexes of 8 age subgroups.

Additionally guided by time of repeat of heat it was analyzed which percentage of the all the accounted was estrous cycles lasted 2–6, 7–12, 13–20 or >20 days.

2.5 Preparation of placental extract

Placental extracts were prepared yourself from sheep placenta by the academician Filatov V.P. method [Filatov, 1949]. Placenta was received during previous lambing of ewes that belonged to the same flock and conformed to following criteria:

- were aged from 3 to 5 years old,
- had normal course of lambing process,
- did not show visible signs of inflammation in reproductive organs, i.e. smell, color and consistency of amniotic fluid and afterbirth meet norm,
- did not show clinical signs of systemic disease,
- in anamnesis did not have abortions and stillborn,
- did not have solid subcutaneous structures in head and throat (suspicion of actinomycosis).

Placenta was obtained in final phase of parturition in a sterile plastic bag. Procedure for manufacture of placental extracts included following steps:

- washing of placenta with sterile saline solution;
- exposure of material in dark place for 5 days at a temperature of 4–6 °C;
- shredding of placenta to pieces sized no more 0.5×0.5×0.5 cm;

- addition of saline solution in the ratio 1:5, exposure of mixture for 1 hour at room temperature;
- exposure of mixture in a water bath at a temperature of 90 °C for 5–10 minutes;
- filtering of mixture through a cotton-gauze filter and packaging;
- sterilization of packaged extract by autoclaving at 1 atmosphere and a temperature of 120 °C for 1 hour.

The made extracts were tested for sterility by seeding on meat-peptone agar and stored before use in the refrigerator at a temperature of 4–5 °C.

2.6 Statistical analysis

The research results were estimated statistically with common accepted algorithms [Lakin, 1990] using mathematical tools of program "Excel" of package "Microsoft Office 2010".

3. Research results

The obtained results had confirmed positive effects of procedure of pre-treatment of anestrus ewes with ovine placental extract to improve the renewal of their sexual activity – proportion of sheep showed signs of heat (E%) and index of heat (IH_i) of all three experimental groups were higher in comparison with the control (Tab. 2). E% was the highest with injections of placental extract only and after complement with oestrogen or neurotrophin decreased. Index of heat (IH_i), that takes into account not only heat fixed during insemination period but and cases of parturition after free mating in "add-mating period" too, for animals of all the experimental groups was highest indicating that all the ewes had heat.

Table 2. Reproductive traits and indexes after use of experimental scheme of preparing of anestrus ewes to insemination campaign

№	Animal group	N/n*	Traits			
			E%, %	F%, %	Pf, lamb/ewe	Fm%, %
1	"PE"	7/22	100.0±0.0 ^b	100.0±0.0 ^b	1.05±0.05 ^b	104.8±5.1 ^a
2	"PE+F"	6/18	88.3±9.1 ^{a,b}	96.7±3.7 ^b	1.33±0.18 ^{a,b}	130.0±20.0 ^a
3	"PE+Pr"	7/25	90.8±5.3 ^{a,b}	97.6±2.6 ^b	1.08±0.06 ^{a,b}	105.2±6.7 ^a
4	"C"	8/285	84.8±2.4 ^a	85.9±2.9 ^a	1.21±0.05 ^a	104.8±5.9 ^a
			Indexes			
			IH _i , y.o.	IHT _i , y.o.	ICT _i , y.o.	IL _i , y.o.
1	"PE"	7/22	1.00±0.00 ^b	1.33±0.16 ^a	1.13±0.10 ^b	1.05±0.05 ^a
2	"PE+F"	6/18	1.00±0.00 ^b	1.36±0.17 ^a	1.31±0.12 ^{a,b}	1.13±0.13 ^a
3	"PE+Pr"	7/25	1.00±0.00 ^b	1.33±0.14 ^a	1.33±0.19 ^{a,b}	1.05±0.07 ^a
4	"C"	8/285	0.94±0.01 ^a	1.49±0.04 ^a	1.61±0.09 ^a	1.04±0.06 ^a

Notes. 1) N – number of age subgroups, n – number of animals in groups; 2) Parameters in the same column with different subscripts differ with P>0.95 (p<0.05).

The use of placental extract accelerated onset of the first heat about something is evidenced the decreasing of index IHT_i of all the research groups.

Injections of extract to anestrus ewes positively influenced on fertility (F%) and contributed to reduce the index ICT_i . Approximation of value ICT_i to 1 shows that number animals that became pregnant during the first heat had increased.

In contrast to other traits prolificacy (Pf) and index of lambing (IL_i) of experimental groups weren't absolutely higher than control and depended from the type of additionally injected substances – the most prolificacy was noted in group with addition of "Folikulin" "PE+F", the lowest – in group "PE", the largest index of lambing – in "PE+F", the lowest – in control group. Increasing of fertility (Fm%) versus control was noted in group "TP+F" only.

Thus, double treatments of ewes with placental extract in non-breeding season had prolonged effect that was showed by acceleration and improvement of sexual activity, increasing of fertility and decreasing of prolificacy against similar parameters of untreated control animals. Addition to scheme the substance of estrogenic action increased prolificacy but reduced the positive effect of the extract on density of appearance of heat. Additional injections of neurotropic substance "Prozerin" worsened density of appearance of heat and did not eliminate the negative effects of placental extract on prolificacy.

After analysis of indexes that characterize the revival of sexual activity by individual animals it was suggested that the use of the extract improved proliferative processes in genital tissue that accelerated onset of heat and promoted formation of better quality ova.

Results of display by ewes of repeated heat showed that in control group were present cycle with duration of all ranges, and proportion of cycles more 20 days only slightly conceded to shorter (diagr. 1). The cycles longer than 20 days were found in all the experimental groups, but repeat of heat after 2–6 days were fixed in "PE+Pr" only.

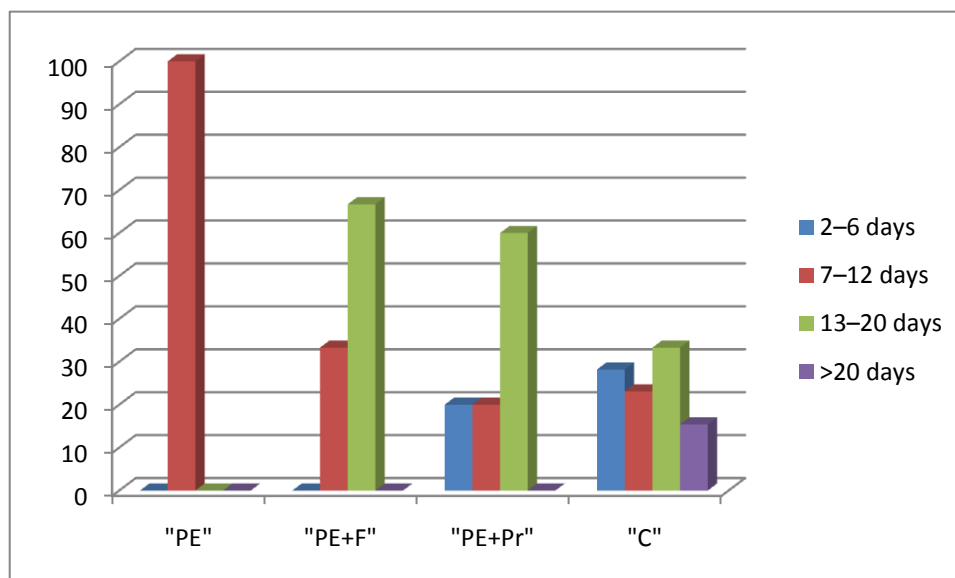


Diagrama 1. Distribution of sexual cycles according to their duration.

4. Discussion

Use of placenta has a long history in folk medicine of eastern and some western nations. Preparations of placenta contain amino acids, peptides, lipids, vitamins, minerals [Lomakin, Artsymovich, 1991; Torshin, Gromova, 2012], glucosaminglicans [Chicalo, Kvahadche 1972] glutamate [Gupta, Chattopadhyay, 2009], polynucleotides [Kvahadze, Harnytskaya, 1972], antioxidants [Togashi et al., 2002], reduced forms of NADH and NADPH [Datta, Bhattacharyya, 2004]. Also it was diagnosed the presence of hormonal [Chicalo, 1972] and neurotropic [Zholnerovych, 1977; Torshin, Gromova, 2012] substances. With human and animal models the ability of placental extracts enhance the cellular and humoral immunity was demonstrated [Chakraborty et al., 2006; Park et al., 2011]. The positive impact of placental preparations is associated with significant content and diversity of active biological substances. While in modern medicine the use of placental preparations especially in Western countries is limited, in veterinary medicine they are used widely [Darycheva, Ermolaev, 2011] especially for treatment of gynecological disorders [Nikitina, Kacharyan, 2013].

Placental extract per se is a one of types of tissue preparations. The lasts are conventionally classified according to nature of solvent: water or alcohol. Depending on type of solvent and method of obtaining the content of bioactive substances in preparations varies [Chicalo, 1972; Gupta, Chattopadhyay, 2009; Park et al., 2011]. To aqueous types it belong the extracts manufactured by method of academician Filatov [Filatov, 1949]. However, unlike extracts that made with consecutive cleaning of aqueous solutions of fresh obtained ground material, as important active components of tissue preparation manufactured by the Filatov's method are considered the biogenic activators, which formed in tissues exposed to adverse conditions [Filatov, 1951]. Nature of these activators is not completely understood. It may be as a simple matter and components more complex in structure. Thus, placental extracts manufactured by the Filatov's method in addition to substances that are typical for placenta contain factors that can act as triggers of nonspecific biochemical and physiological processes.

In present work the aqueous extract of placenta which is produced by the Filatov's method was used. In view of the above, the revealed positive effect of applied schemes may be explained by the several factors. At first, components of extract are able to activate and enhance individual immunity, which helps to treat latent inflammation and improve acid-base balance in horns at the time of insemination. The latter influences positively on viability and transport sperm and is supported indirectly by increased fertility and reduced IH_i and ICT_i in animals of all the experimental groups.

Secondly, the factors of positive impact of extract may become biogenic activators, duration of extract action and choice of timing of treatment. In experiments with ewes, rams and lambs we found that the effect of the placental extract stores till 20 days after last injection. The post-mortal and laparoscopic inspections showed that in early August in ovaries of Ascanian Merino sheep a natural activation of folliculogenesis happens with formation a lot of different diameter follicles [Lobachova, 2016]. It may be assumed that processes initiated

by treatment with placental extract is synchronized with start of natural recovery of folliculogenesis and this synergy accelerated renewal of sexual activity and promoted ovulation of more developed ova.

Third, some researchers suggest that positive effect may do hormones contained in the extract including of estrogenic nature [Chicalo, 1972]. But amount of estrogen in placental extracts is insignificant [Beyssac et al., 1986]. In addition, number of receptors for estrogen in ovaries and pituitary gland [Wise et al., 1975] of anestrous sheep is reduced. Also it was showed that some components of placental extract are able to inhibit aromatase activity of granulosa cells [Al-Gabory et al., 1995]. So it may hardly claim a noticeable effect of estrogen extract on folliculogenesis in our experiment. However, in additional injections of exogenous estrogen in amount that exceeds its content in placental extract, hormones may long-store in body and sensitize some parts of the hypothalamus to increase secretion of gonadotropin-releasing factors. The latter, in turn, may stimulate the pituitary to increase excretion of gonadotropins, enhance steroidogenesis and improve the development of follicles in ovaries. Results of group "PE+F" is certain confirmation of this assumption.

Absence of impact of substance with anticholinesterazal activity, in our opinion, should explain by short time of action due to rapid neutralization by organism. It is known that changing the ratio of activity of sympathetic and parasympathetic chains of nervous systems can activate and improve the functionality of genital organs of animals [Yun, Lee, 2005]. But to achieve this neurotropics must inject daily for several days. In our experiment we limited number of injection to two and their number was not enough. However, taking into account the raising in labor costs with increasing of number of injections, magnification of amount of procedures may not be considered as economically justified.

Published data indicate that estrous cycles with 2–5 days length often are registered at beginning of contact ewes with rams [Martin et al., 1986]. It is supposed that the cause of such short cycles is inability of follicular cells remained in place of ovulation transform to fully developed luteal and thus adequately prevent the development of new follicles, inhibit the synthesis of estrogen and prostaglandins and new ovulation [Chemineau et al., 2006]. Cycles lasting from 9 to 12 days are often recorded in beginning of renewal of sexual activity and explained by non-full development of follicle and formation of corpus luteum with short term of life [Robinson, 1954; Oldham et al., 1976; Eldon, 1993]. Cycles with extended life-time is associated with delayed resorption of the corpus luteum due to inhibition of prostaglandin synthesis by horn walls. Accordingly, absence in group "PE" and "PE+F" the shortest (2–6 days) and extended (>20 days) cycles indicates that treatment positively influenced on proliferative processes not only in ovaries but in uterus horns too. However, a little number of experimental animals with repeated heat didn't let to accept this as a general pattern.

At last it should be noted that for analysis of efficiency of treatment together with traits that are traditionally used to determine reproduction performance of sheep group (E%, F%, Pf and Fm%), we had applied the individual indexes for

assessment of renewal of sexual activity that determine rapidity of heat display of each animal, time and efficiency of insemination (IH_i , IHT_i , ICT_i). Also distribution of estrous cycles accordingly duration was analyzed. There arises the question of the expedience of such estimating. Of course, for the sheep breeding system in which the general aim is to receive the replacement ewe lambs only (including merino breeds) to characterize the reproductive performance the traditional traits are enough. But in flocks where it is important not only number but also the timing of receipt of lambs (in meat breeds), this approach is justified because gives ability to evaluate the suitability of breed to a particular system of breeding, adaptability to sealing of lambing. Our experiments show that speed of heat display may be controlled artificially and one of a way for this is treatment of anestrus ewes with ovine placental extract only or completed with estrogen. However, the question of further improvement schemes of use of placental extracts in order to maintain its positive effect on rapidity and quality of the first heat and to eliminate negative impact on prolificacy remains.

5. Conclusions

- Double injections of placental extract at a dose of 3 ml per injection per animal with an interval of 5 days in July significantly increase proportion of animals that during next insemination campaign show heat (E%) and fertility (F%), worse prolificacy (Pf), accelerate the display of heat and increase probability of animal to conceive in the first heat, but do not affect fecundity (Fm%) in comparison with control untreated animals.
- Supplement of scheme of placental extract treatment with two injections of the "Follikulin" in amount of 1 ml per injection per animal decreases E%, not affects F%, and increases Pf and Fm%.
- Supplement of scheme of placental extract treatment with two injections of the "Prozerin" in amount of 1 ml per injection per animal does not change the efficiency of placental extract use.

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