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Biotechnological methods for improving reproduction on sheep breeding program using laparoscopic artificial inseminations in Debrecen, Hungary

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SUMMARY

The aim of this study was to evaluate the results of the LAI method in different types of breeds and seasonal variations. Analysing n=536 LAI in 7 years, there were no significant differences observed in the breed in lambing rates. Although the lambing rate of Prolific Merino (F+) was better than any other breed (45.3%). We found significant differences between breeds of Tzigaia, its pregnancy rate was 19.2%. In the case of prolificacy there were significant differences between breeds: Prolific Merino's (FF) was the highest (2.38) and, Indigenous Tzigaia's was the lowest (1.4) from all of them. The obtained results showed that summer inseminations were the most effective in concern of pregnancy rate (49.5%). The results are based on use of the types of semen, showed that using frozen semen was most effective than fresh semen in both parameters, however there were no significant differences. Summarising all tested conditions in our study breeds, seasons and type of semen using LAI the total results of pregnancy rate was 40.3% and the prolificacy was 1.78, respectively. Follow up the out-of-season ovarian, progesterone level of n=36 Prolific Merino were examined and insulin, IGF-1, leptin, T3, and T4 hormones as well, to evaluate the energetic status of the flock. Progesterone analysis showed 43% of examined ewes had a cyclical ovarian function in April and a cyclical ones had good reaction for estrus-induction hormonal treatment. Our results showed that valuable information can be obtained about energy supply from the determination of some metabolic hormones as IGF-1, insulin, leptin.

Keywords: *Booroola gene (FecB); pregnancy rate; prolificacy; IGF-1; ram semen*

INTRODUCTION

Reproductive Biotechnology in animal production is widely used to enhance the production, improve reproductive efficiency and improve the genetic gain (Madan, 2005). Sheep is one of the most important grazing animals possess desirable characteristics for farmers especially inhabiting semi-arid and arid regions such that disease resistance, good acclimation to environmental fluctuations, and endurance to extreme weather (Al-Thuwaini, 2021; Farrag, 2019). Such features of sheep production that contribute to the economic value are important for the developments of livestock production programs (Abd-Allah et al., 2019). Reproductive traits in sheep production such as age at first lambing (Anel et al., 2005; Zegeye et al., 2020), litter size and lambing interval had high economic value (Yavarifard et al., 2015). Therefore, reproductive performance is a critical factor that determining the efficiency of the flock, especially in the developing countries where the sheep industry is significant (Mazinani and Rude, 2020; Ibarra et al., 2000).

Artificial insemination (AI) in sheep is the basic reproductive biotechnological techniques by which, in a combination with technology for fast genetic growth can be succeeded over the application of a limited numbers of top male sires is used (Alvarez et al., 2012). It allows accelerated genetic advancement in commercial flocks, when used in combination with estrus synchronization administration allows births to be planned for a commercially favorable periods(seasons) (Aké-villanueva et al., 2017; Bergstein-Galan et al., 2018). According to the place of semen deposition, there are partial cervical, deep cervical and intrauterine insemination (laparoscopic) (Faigl et al., 2012). The advantage of laparoscopic artificial insemination (LAI) is the possibility of using lower dose of semen (Anakkul et al., 2013), because of the sperm placed nearer to the fertilization site, the problems of passing the cervical barrier can be minimized. The disadvantages are that it requires anesthesia, sophisticated equipment, and a trained person. The mean conception and lambing rates of 44.89% and 44.44% were reported for LAI in a study concerning a big figure of ewes under field conditions (Anel et al., 2005; Zegeye et al., 2020). LAI techniques had well better yields when compared to vaginal or transcervical breeding programs (Casali et al., 2017; El-Badry et al., 2014).

Factors affecting ovulated follicles in ewes are season, nutrition and treatments of estrus synchronization but mostly genetics. Prolific sheep breeds are one of the opportunities on the way to enhance meat production of in the flock. The Australia's Merino has exceptional fecundity due to the present of Booroola (FecB) gene which is an autosomal mutation gene that has a preservative result for ovulation rate. FecB allele was being integrated into a number of sheep breeds in many countries by

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conventional back-cross structure in order to increase the yield. The prolific merino breed announced to Hungary in 1992 comes from crossing the fertile gene-carrying Booroola Merino rams and the local merino breeds (Magyar et al., 1999). The exact locus of FecB gene was discovered on sheep chromosome six (Sousa et.al, 2001), by which it is possible to describe the genotype (homozygous: FF, heterozygous: F+, or not carrier: ++) from blood or semen sample (Magyar et al., 1999).

The fertility is not enough when thawed semen is inseminated in to the vagina or into the uterus via the cervix., because the cooling-freezing-thawing system is a shock for spermatozoa, the capacitation may occur before sperm reaching ova in oviduct. LAI technique was evolved for depositing frozen-thawed semen in the uterine horn of sheep (Magyar et al., 1999). This study aimed to evaluate the effects of the reproductive response of ewes to intrauterine LAI technique taking account with the breed of ewes, the dose of semen, types of semen (fresh vs. frozen) and time of insemination (season).

MATERIALS AND METHODS

Description of the farm and animal management

The laparoscopic artificial inseminations were done on the sheep farm of Agricultural Faculty of Debrecen University in Kismacs between 2002 and 2009. A total of 536 ewe breeds aged between 2 to 7 years old, with a body condition score 2.5 to 3 were used in the experiment both in breeding and out of the breeding season. The breeds of inseminated ewes were Merino, Prolific Merino, Texel, Indigenous Tzigaia, and Milking Tzigaia. The Prolific Merinos were homozygous (FF) vs. heterozygous (F+) Fecundity Booroola gene carrier's ewes. Animals eat grass, alfalfa hay ad libitum and a 0.4 kg mixture of maize-oat-rye /day /ewe during the flushing period. All animals were deprived of feed and water for 12 hours before insemination and experimental procedures were carried out according to European regulations on the Care and Welfare of Animals. Chrono-Gest intravaginal sponges A.U.V (MSD Animal Health) were used for a 13 days' treatment. Folligon injection A.U.V 300IU, 500IU and 600IU pregnant mare serum gonadotrophin (PMSG) at the time of sponge withdrawal was administered. LAI was performed 60 h after sponge removal.

Semen preparation

Fresh ejaculates sperm from fertile rams were collected and assessed for volume, mass motility and concentration. The sperm dilution ratio depended on the results of assessment. The diluted sperm were put to a sperm transport- container (Equitainer) and transported to Debrecen city (150 km from the place of collection). The diluted sperm was used for insemination within 48 hours.

The frozen-thawed ram semen was stored in liquid nitrogen in special containers and was microscopically evaluated before insemination for its motility and viability. The straws were thawed in water bath at 37 ºC for 30 seconds at a time of insemination.

Laparoscopic artificial insemination

After 12 h starvation the ewe was prepared for a minor surgical procedure under local anaesthesia, and was placed to laparoscopic cradle with the hindquarters elevated of 45º. The insemination was done by a highly skilled veterinary specialist. The semen was deposited in both uterine horns with an insemination pipette. After laparoscopic AI, all ewes had received antibiotics (Betamox LA A.U.V) and non-steroid anti-inflammatory drugs (NSAIDS).

Pregnancy rate and prolificacy

The pregnancy rate and prolificacy per ewes were determined by using the following equation:

Pregnancy rate (%) = (Number of ewes lambed / Number of ewes inseminated) x100

Prolificacy (Nr) = Number of lambs born/ Number of ewes lambed

Following up the out of season hormonal function of the ovary and the energetically status of Prolific Merino ewes

An examination was done if what is the rate of the Prolific Merino ewes with cyclical ovarian function in out-of–season reproductive period and which hormones are suitable to value the energetic status of the flock, respectively. In the spring-early summer period of the year, n= 37 Prolific Merino (FF and F+) ewes in different ages and conditions were analyzed. The first blood sample for progesterone analysis was taken at the end of April and flushing was started at the same time (grass, alfalfa hay ad libitum and a 0.4 kg mixture of maize-oat-rye/day/ewe). The second sampling was 11 days later. Ovarian function was considered cyclical (presence of CL) if an elevated (≥1.00 nmol/l) progesterone level was found in at least one of the two blood plasma samples.

At the same time as the second sampling, an 11-day progestagen treatment with Chrono-gest (30 mg FGA) vaginal sponge was started, and 625 IU PMSG (Folligan inj.) was injected intramuscularly at sponge removal.

From blood samples insulin, insulin-like growth factor-1, thyroxine (T4) and triiodothyronine (T3) were analyzed for following up the energy supply and leptin for controlling the nutritional status of animals.

Blood samples were collected into heparinized tubes by puncturing the vena jugulars. Then after centrifugation at 2000rpm for 15 minutes 1.5 ml of plasma was taken to plastic tubes and was stored at -20 ºC until assayed. The hormonal analysis was done in Endocrinological Laboratory of Szent István University Faculty of Veterinary, Budapest.

Data analysis

The effects on the observed reproductive parameters of the breeding farms (n=536) and the hormone analysis' values of experimental group (n= 36) were analyzed using SPSS statistics 26. Values were

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considered significant with p<0.05. The results were evaluated with chi-square test completed with Yates-correction, with Student t-test and ANOVA analysis respectively.

RESULTS AND DISCUSSION

In *Table 1* shows the pregnancy rate and prolificacy according to the type of semen, the breed and the month of laparoscopic insemination.

Table 1. **Pregnancy rate and prolificacy according to the type of semen, the breed, and the month of laparoscopic insemination**

Type of semen	Breed	Number of inseminated ewes	Month of LAI	Number of ewes lambed	Number of lambs born	Pregnancy rate (%)	Prolificacy
FRESH	Merino	14	January	8	13	57.14	1.62
	Prolific merinoF+	38	January	11	15	28.94	1.36
	Prolific merino FF	17	January	4	11	23.52	2.75
TOTAL		**69**		**23**	**39**	**33.33**	**1.69**
FROZEN	Indigenous Tzigaia	14	February	6	8	42.85	1.33
		28	May	4	6	14.28	1.50
		10	September	0	0	0	0
	TOTAL	52		10	14	19.23	1.40
	Milking Tzigaia	22	September	7	13	31.81	1.85
		23	October	10	16	43.47	1,6
	TOTAL	45		17	28	37.77	1.64
	Texel	7	January	2	2	28.57	1
		34	October	16	29	47.05	1.81
	TOTAL	41		18	31	43.90	1.72
	Merino	25	January	7	8	28.00	1.14
		27	May	12	18	44.44	1.5
		11	June	4	10	36.36	2.5
		10	July	5	11	50.00	2.2
		34	September	14	29	41.17	2.07
	TOTAL	107		42	76	39.25	1.80
	Prolific merino F+	19	January	7	10	36.84	1.42
		28	May	16	32	57.14	2.00
		22	June	11	18	50.00	1,63
		23	July	12	16	52.17	1.33
		12	August	7	13	58.33	1.85
		27	September	12	24	44.44	2.00
		11	October	6	7	54.54	1.16
	TOTAL	143		71	120	49.65	1.69
	Prolific merino FF	14	January	4	13	28.57	3.25
		14	May	5	12	35.71	2.4
		10	June	5	14	50.00	2.8
		11	July	6	10	54.54	1,66
		17	September	9	18	52.94	2.00
		13	October	6	15	46.15	2.5
	TOTAL	79		35	82	44.30	2.34
TOTAL		**467**		**193**	**323**	**41.32**	**1.67**

Pregnancy (%) = (Number of ewes lambed / Number of ewes inseminated) x100; Prolificacy (Nr): Number of lambs born per ewes lambed. Nr= number

Effects of breed on pregnancy rate and prolificacy

Table 2 shows the pregnancy rate and the prolificacy of different breeds using LAI methods. There were no significant differences observed in the breed between Texel, Merino, Prolific Merino/FF, Prolific Merino F+ and Milking Tzigaia in pregnancy rates. Although the pregnancy rate of Prolific Merino (F+) ewes was better than any other breed (45.3%). We found significant differences between ewe breeds of Indigenous Tzigaia (p<0.005), its pregnancy rate was 19.2%. In the case of prolificacy there were significant differences between breeds: Prolific Merino's (FF) was the highest (2.38), and, Indigenous Tzigaia's was the lowest (1.4) from all of them. (*Table 2*).

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Table 2. **The pregnancy rate and the prolificacy of different breeds using LAI methods**

Breeds of ewe	Number of Inseminated ewes	Number of lambed ewes	Number of lambs born	Pregnancy rate (%)	Prolificacy
Indigenous Tzigaia	52	10	14	19.2*	1.4*
Milking Tzigaia	45	17	28	37.8	1.64
Texel	41	18	29	43.9	1.61
Merino	121	50	89	41.3	1.78
Prolific Merino F+	181	82	135	45.3	1.64
Prolific Merino/FF	96	39	93	40.6	2.38*

Pregnancy rate (%) = (Number of ewes lambed / Number of ewes inseminated) x100; Prolificacy (Nr): Number of lambs born per ewes lambed.
* Significant differences ($p < 0.05$) between the data within the column

The effect of ewe breed on pregnancy rate after an AI program has been found to be significant in many literatures. A pregnancy rate of 42.10% was recorded for Lacaune Sheep breed on application of laparoscopic artificial insemination using frozen-thawed semen (Zegeye et al., 2020). Karagiannidis et al. (2001) found that the lambing rate was higher in the Chios breed (53.1%), than the Vlachiki breed (38.2%) and crossbred (Vlachiki x Chios) (47.6%) ewes, respectively.

In addition, rams can significantly affect fertility rates after LAI (Perkins et al., 1996; Anel et al., 2005). Windsor et al. (1997) reported significant changes in pregnancy rate of ewes fertilized with different ram sperm. However, Zegeye et al. (2020) showed that the ram effect did not show a significant difference between the same breeds in terms of pregnancy and proficiency rates. Ewe fertility varies significantly after AI due to differences in cervical morphometric characteristics between ovulation and ovulation rate. Morphometric studies were performed on four breeds (Assaf, Churra, Castellana and Merino) and showed significant differences in the number of folds, length, width and distance between folds, as well as breed variations in the depth of penetration of the catheter into the cervix during AI (Naqvi, 1998; Fukui and Roberts, 1978; Halbert et al., 1990). There are differences in the physico-chemical properties of cervical mucus, which can make it difficult to determine the movement of sperm (Kaabi et al., 2006). Therefore, LAI is an excellent technique for improving the birth rate and genetic value of the sheep stock (Fukui et al., 2010; Palacin et al., 2012).

Effects of season on pregnancy rate and prolificacy

Summarizing all tested conditions (7 years, different sheep breeds, seasons and type of semen) the total results of LAI on pregnancy rate was 40.3% (216/536) and on the prolificacy was 1.78 (385/216), respectively (*Table 3).* Despite our expectations, our results showed that summer inseminations were the best in concern of pregnancy rate (49.5%). The summer and the autumn pregnancy rates were significantly higher than the winter and spring ones. Even the prolificacy was the highest (1.91 lamb/ewe) after summer LAI, however there were no significant differences between the seasons in this regard (*Table 3*).

Table 3. **Effects of season on pregnancy rate and prolificacy using LAI method**

Time of LAI Month / Season	Number of Inseminated ewes	Number of ewes lambed	Number of lambs born	Pregnancy rate (%)	Prolificacy
January	123	42	67	34.2	1.59
February	14	7	13	50	1.86
Winter	**137**	**49**	**80**	**35.8**	**1.63**
May	97	38	67	39.2	1.76
Spring	**97**	**38**	**67**	**39.2**	**1.76**
June	43	21	40	48.8	1.90
July	45	22	33	48.9	1.50
August	23	12	24	52.2	2.00
Summer	**111**	**55**	**97**	**49.5***	**1.76**
September	98	36	72	36.7	2.00
October	83	38	69	45.8	1.82
Autumn	**181**	**74**	**141**	**40.8***	**1.91**
Total	**536**	**216**	**385**	**40.3**	**1.78**

Pregnancy rate (%) = (Number of ewes lambed / Number of ewes inseminated) x100; Prolificacy (Nr): Number of lambs born per ewes lambed lambed. Nr= number, * Significant differences ($p < 0.05$) between the data within the column)

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Sheep reproduction is seasonal, the season starts from mid-March and end in late July or early August, characterized by the absence of ovulation and sexual behavior (Hristova et al., 2011). In our study the pregnancy rate of spring LAI (May) was lower than summer or autumn results. Anel et al. (2005) described the maximum fertility in July, August, September and October (58.3%, 58.1%, 61.9% and 65.2%), respectively. We also registered the best fertility rate after summer insemination in June, July August, and October (48.8 %; 48.9%; 52.2% and 45.8%). Our result as shown in Table 1 supported by Novotni-Dankó (2003), originally the reproduction in ewes is seasonal, but nowadays many breeds of sheep are able to conceive in out-of-season periods as well.

Effects of the use of fresh and frozen semen on pregnancy rate and prolificacy

These results are based on the types of semen for LAI showed that using frozen semen had better results than using fresh semen both pregnancy rate and prolificacy (*Table 4*), however there were no significant differences.

Table 4. **Effects of the use of fresh vs. frozen semen on pregnancy rate and prolificacy**

Types of semen	Number of inseminated ewes	Number of lambed ewes	Number of lambs born	Pregnancy rate (%)	Prolificacy
Fresh	69	23	39	33.3	1.69
Frozen	467	193	346	41.3	1.79

Pregnancy rate (%) = (Number of lambs born a live / Number of inseminated ewes) x100; Prolificacy (Nr): Number of lambs born per ewes lambed. Nr= number; There is no significant differences (p > 0.05) both types of semen in the fertility result

There are both fresh and frozen semen inseminations by laparoscopic in sheep breeding programs (Killeen et al., 1982; Maxwell 1984). Semen cryopreservation has a negative effect, that damage the sperm cells growing the population of non- viable sperm cell (Jiménez-Rabadán et al., 2012), but according to Salamon and Maxwell (2000), only 20-30% remains biologically safe, even though, may not affect the motility of the spermatozoa the membrane of spermatozoa was changed and decreases cell survival and fertilization potential. Due to ultrastructural change of sperm membrane the fertility rates of frozen thawed semen are low. There is a redistribution of lipids that changes lipid–lipid and lipid–protein relations, which are necessary for the normal function of sperm membranes (Park and Graham, 1992). Biochemical and functional changes undergone as well, which leads to insufficient movement and loss of viability of these spermatozoa in the genital tract (Salamon and Maxwell, 2000). Even though, satisfactory fertility result can be got with fresh semen using other techniques of insemination, whereas LAI used mostly for frozen-thawed semen. However, many approaches of insemination with profitable use and satisfactory results have been developed, the use of frozen sperm is not very common, as this process is rarely used in field applications (Salamon and Maxwell, 2000; Moses et al., 1997; Anel et al., 2003) In this study the results of LAI with frozen semen was better than with fresh, but there were no significant differences between them or the pregnancy rate or the prolificacy.

Following up the out- of- season hormonal function of the ovary and the energetically status of prolific merino ewes

Bsed on the results of progesterone analysis of two blood samples collected 11 days apart, 21 ewes (57%) had acyclical and 16 (43%) had cyclical ovarian function in out-of-season period.

There were no significant differences of age of experimental animals. Nevertheless, the average body weight of cyclical ewes was significantly higher (P<0.001) at the beginning and at the end of the flushing than the acyclic ones. According to Geenty et al., 2013, because ewes need to be in good body weight and body condition (BCS) for high ovulation rates at mating. However, their body weight gains during flushing did not differ significantly *(Table 5).*

After progesterone + PMSG treatment, laparoscopic ovulation rate (OR) examination showed only 3 animals had no corpus luteum (CL) on the ovary. It suggests that the acyclical ones had a good reaction for estrus-induction hormonal treatment.

After LAI the pregnancy rate was 43.8% of those animals which had formerly cyclical ovarian function. On the other hand, there became only 1 pregnant among the acyclic ones (pregnancy rate: 4.8%), which is significantly lower performance (*Table 6*).

Comparing the dates of the ewes with acyclic and cyclical ovarian activities, the ewes which had cyclical ovarian activity had higher leptin level and significantly higher IGF-1 and insulin concentration than acyclic ones (*Table 6*). This result supported Beam and Butler (2019): the level of IGF-1 accurately reflects the energetic status of the body and in sheep, the local concentration of IGF-1 has great importance for the morula-early blastocyst-stage embryo developing in the fallopian tube and uterus (Watson et al., 1999).

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Table 5. **The age, weight and pregnancy data of ewes cyclic and acyclic ovarian function in out-of-season period (April)**

Parameters		**Ovarian activity**	
		Acyclic, n=21 (57%)	**Cyclical, n=16 (43%)**
Age (month) at the beginning of experiment (in April)	Mean ± SD	47.5 ± 29.4	57.1 ±24.1
Weight, kg, at the beginning of flushing (in April)	Mean ± SD	32.3±3.6	38.0* ±5.1
Weight, kg, at the end of flushing (in May)	Mean ± SD	34.9± 5.1	40.8*± 5.1
Weight gain, kg	Mean ± SD	2.6 ±1.4	2.9 ±1.4
Lambed (Nr)		1	7
Pregnancy rate (%)		4.8	43.8*

SD= standard Deviation. * = significant differences ($p < 0.05$) between rows' data

Table 6. **The plasma level values of some metabolic hormones on 11th day of flushing of acyclic and cyclical ewes**

Hormone		**Ovarian activity**	
		Acyclic (n=21)	**Cyclical (n=16)**
Leptin, ng/ml HE	Mean ± SD	2.05±0.30	2.19±0.35
IGF-1, nmol/l	Mean ± SD	19.95±4.38	22.57*±4.26
Insulin, IU/L	Mean ± SD	20.98±4.74	24.44*±3.56
T3 (nmol/l)	Mean ± SD	1.21±0.12	1.26±0.11
T4 (nmol/l)	Mean ± SD	104.98±16.47	111.36±14.88

The plasma level values of some metabolic hormones on 11th day of flushing of acyclic and cyclical ewes. * = significant differences, where, SD= standard deviation

The feeding can be influence reproductive designs as Márton et al. (2009) noticed raised insulin and IGF-I levels in Awassi ewes cyclic before energy supplementation compared to acyclic ones. It supports Gordon's (1999) theory, that body fat content directly effects on hypothalamic activity and GnRH secretion and on hypothalamic-pituitary sensitivity to ovarian hormones, respectively.

CONCLUSIONS

In conclusion, for the proper regulation of the reproduction process, it is necessary to increase the productivity of the flocks which affects the productivity of different sheep breeds higher in different climatic areas.

Some of the factors are breeding, season; types of semen, dose of semen and ovulation are important part of effective reproductive management practice in sheep production systems that allows designing and leading the breeding and pregnancy operation in a short period of time.

The Prolific Merino sheep breed (FF and F+) had higher fertility when compare to Indigenous Tzigaia sheep breed because of the present of Booroola (FecB) major gene. Sheep fertilized in spring, autumn and summer had an advanced fertility rates in contrast sheep inseminated in winter, they are seasonally breeding animals so as in winter the fertility rate is less. In our study better fertility with frozen semen was achieved than fresh semen (41.32% vs. 33.33%) using laparoscopic insemination.

Following up the ovarian activity in out – of – season (April–May) reproductive period, 43% of examined Prolific Merino ewes had cyclical ovarian function. The acyclic ones give good reaction for cycles-induction, the formerly cyclic ewe's conception was significantly higher than acyclic ones. Determination of some metabolic hormones as IGF-1, insulin, leptin can give valuable information about energy-supply. The success of cycles induction depends on energy supply of the animal. In general, evaluation of sperm type, genetics of sheep, energy supply of ewes are key elements that can lead to more consistent results, help the spread of LAI use on commercial sheep farms.

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CONFLICT OF INTEREST

There is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript. The laparoscopic insemination process was an everyday practice included in the farm's operation plan. The process was carried out based on the act of

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"XXVIII of 1998. Law on the Protection and Welfare of Animals" passed by the Hungarian Parliament. 9§ (1): Interventions involving pain or damage to the animal may only be performed by a person with a specialized qualification or, in the case of interventions common among animal keepers, only with experience." The inseminations were done by veterinary medicine Károly Magyar (co-author of the manuscript), who has a special qualification, and a large skill in laparoscopic technique. The experiment which included blood sampling from 37 animals was carried out by the same veterinary and in accordance with the regulations of the aforementioned law.

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