

## Efficiency analysis of standard and day 0 superovulatory protocols in Boer breed goats

**Juraj Grizelj<sup>1</sup>, Branimira Špoljarić<sup>1</sup>, Tomislav Dobranić<sup>1</sup>, Martina Lojkić<sup>1</sup>,  
Fernando Sánchez Dávila<sup>2</sup>, Marko Samardžija<sup>1</sup>, Foteini Samartzi<sup>3</sup>,  
Iva Getz<sup>1</sup>, and Silvijo Vince<sup>1\*</sup>**

<sup>1</sup>*Clinic for Obstetrics and Reproduction, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia*

<sup>2</sup>*Autonomous University of Nuevo Leon, Faculty of Agriculture, Laboratory of Animal Reproduction, Marín, Nuevo Leon, Mexico*

<sup>3</sup>*Veterinary Research Institute, Hellenic Agricultural Organization, Themi-Thessaloniki, Greece*

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### ABSTRACT

The aim of this research was to investigate the ovarian influence on the success of two superovulatory protocols in goats. Fifteen goats were used twice in the study in a crossover design: once within the control group where standard protocol was applied, and the other time in a modified, day 0 group, where no dominant follicle was present and the follicular cohort was homogenous ("Day 0 protocol"). All the animals were synchronized with vaginal pessaries and superovulated with 6 decreasing doses of pFSH. Although the day 0 protocol significantly decreased the presence of the dominant follicle before the start of the superovulation treatment; it did not improve the superovulatory success. Fifty-four percent of goats in the control group had a dominant follicle which exerted a negative influence on the number of transferable embryos. The other 46% of goats from the same group had a very good superovulatory response, which improved the overall response of the control group in comparison with the day 0 group. Another reason for the lower superovulatory success of the day 0 protocol was the smaller number of second and third category follicles (3.5 - 5.5 mm in size) at the onset of the superovulation protocol, that positively correlated with the number of the transferable embryos ( $r=0.67$ ;  $P<0.05$ ). In Boer goats, for the success of superovulatory treatment, the number of follicles of a certain size before the start of the superovulatory treatment has an effect as important as the effect of dominance. The application of the FSH hormone should start at the time when a suitable number of follicles belonging to the second category are present on the ovaries when performing the day 0 protocol.

**Key words:** goat, superovulation, dominant follicle, follicular population, embryo, superovulatory success

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\*Corresponding author:

Silvijo Vince, Clinic for Obstetrics and Reproduction, Faculty of Veterinary Medicine, University of Zagreb, Vjekoslava Heinzela 55, 10000 Zagreb, Croatia, Phone: +385 1 2390 185; E-mail: svince@vef.hr

## Introduction

Goats are seasonal polyestric animals in mild climate regions, with the breeding season becoming longer towards the equator (ZARROUK et al., 2001). A year-round breeding season has also been observed in the Boer goat in mild continental climate regions (ĐURIČIĆ et al., 2009a), indicating that this kind of reproductive activity could be hereditary rather than environmentally influenced (DJURICIC et al., 2011). However, paragenetic factors such as dietary regime, should also be taken into account, particularly macroelements such as calcium and phosphorous serum levels (SAMARDŽIJA et al., 2011). Further, ĐURIČIĆ et al., (2012) reported that Boer goats, after they were introduced from the southern to the northern hemisphere, gradually became acclimated to a new seasonality. A higher percentage of live-born offspring were expected in Croatia during the optimal breeding season than in most European goat breeds (ĐURIČIĆ et al., 2009a, 2009b).

Despite the progress achieved in goat breeding, commercial hormone preparations and protocols, the superovulatory response in goats is still characterized by high variability. The causes may be related to the sexual cycle phase and the influence of the ovary function (LINDSELL et al., 1986; WANI et al., 1992; ĐURIČIĆ et al., 2008). TASDEMIR et al. (2011) found that transferable embryo yield is affected by the breed while AYRES et al. (2012) found that superovulation can be reliably produced in goats, even without an exogenous source of progesterone.

The presence or absence of a dominant follicle is one of the factors that determines the success of the superovulatory response, as it affects the distribution of the follicular cohort and determines the ovulation percentage, as well as the number of embryos obtained (GONZALEZ-BULNES et al., 2000, 2003a, 2003b; MENCHACA et al., 2002; RUBIANES and MENCHACA, 2003; MENCHACA et al., 2007; GINTHER and KOT, 1994; DE CASTRO et al., 1999).

Concerning goats, there are records about the effect of the number of follicles ranging in size from 2-6 mm present at the start of the treatment on the percentage of ovulation, and those measuring 4-6 mm on the number of obtained embryos (GONZALEZ-BULNES et al., 2003a). Studies pertaining to ovarian factors show the significant effect of dominant follicles measuring 6 mm or more.

The aim of this research was to evaluate superovulatory response in Boer goats that were submitted to two different superovulatory protocols (standard and day 0 protocols) and to assess the role of ovarian factors - the follicular population and the presence of a dominant follicle at the start of superovulation treatment.

We hypothesize that the application of gonadotropin one day following ovulation could optimize superovulatory response in situations when there is no dominant follicle

present but only a uniform follicular population starting to grow in the first wave (day 0 protocol).

### Materials and methods

Fifteen Boer goats aged 1 to 3 years and  $41.0 \pm 10.5$  kg of live weight were included in the study, which was conducted during the breeding season (latitude  $45^\circ\text{N}$ ). The animals were fed with hay and concentrate twice a day and water was available *ad libitum*.

The animals were randomly divided into two groups: the control (standard protocol - C) and the day 0 group (experimental protocol - D0). Six to twelve weeks later, the protocol and the group were switched in a crossover design, so that every animal was submitted twice to superovulatory protocols. The superovulatory response was considered unsuccessful if early luteal regression or cysts were found, or if no ovulation occurred. Such animals were removed from further statistical analysis.

In order to analyse the influence of a dominant follicle, the results were additionally divided into two groups, depending on whether or not at the start of the superovulation a dominant follicle was present (dominant follicle - DF and, no dominant follicle - NO\_DF).

*Day 0 group.* The goats were submitted to oestrus synchronization using vaginal progestagen pessaries containing 40 mg fluorogestone acetate (FGA) (Chronogest<sup>®</sup>, Intervet, Lima, Peru) during 11 days. Forty-eight hours before the pessaries were withdrawn, 250 IU of equine chorionic gonadotropin (eCG) (Folligon<sup>®</sup>, Intervet, Boxmeer, The Netherlands) and 50  $\mu\text{g}$  of cloprostenol (Estrumate<sup>®</sup>, Schering-Plough, Friesoythe, Germany) were applied intramuscularly (i/m).

Twenty-four hours after pessary withdrawal, the detection of oestrus was performed every 4 hours until positive detection occurred. Induction of ovulation was done 12 hours following positive oestrus detection by i/m application of 8.4  $\mu\text{g}$  of a gonadotropin releasing hormone (GnRH) analogue, buserelin acetat (Receptal<sup>®</sup>, Intervet, Boxmeer, The Netherlands).

Forty-eight hours after GnRH analogue application, a superovulation protocol that consisted of 6 i/m doses of porcine follicle-stimulating hormone (pFSH) (Folltropin<sup>®</sup>, Vetrepharm, Belleville, Canada), injected every 12 hours (50 mg + 50 mg, 25 mg + 25 mg, 25 mg + 25 mg) was applied. After the fifth and sixth pFSH injection, cloprostenol, at a dose of 50  $\mu\text{g}$  was applied. The following day - i.e. 24 hours after, oestrus detection was performed every 4 hours using an adult buck wearing an apron. If oestrus was detected, the goat was mated individually twice a day at an 8-10 hour interval, and the following day if oestrus was still detectable. The time frame was recorded in which oestrus was detected following the fifth dose of pFSH and cloprostenol injection, as well as the duration of oestrus.

*Control group.* Progestagen pessaries were inserted and left for 11 days. A single injection of 50 µg of cloprostenol was applied on day 9 following the insertion of the pessaries, in the morning, simultaneously with the superovulatory treatment, which was the same as described previously. Oestrus detection and mating were performed as described previously. The time frame in which oestrus appeared following the pessary removal was recorded for each animal, together with the duration of oestrus.

*Treatments designed for all goats included in the study*

*Laparotomy for uterine flushing and the anaesthetic protocol.* The ovulations were confirmed during ultrasound examinations the day before uterine flushing by detection and counting of CLs. Uterine flushing was performed by laparotomy, 7 days after the first mating (D7). Anaesthesia was induced with 0.11 mg/kg of xylazine (Xylapan<sup>®</sup>, Vetoquinol) i/m followed by an i/m injection of 5.5 mg/kg of ketamine (Narketan<sup>®</sup>, Vetoquinol) 10 min later.

The does were placed in a dorsal recumbent position on an inclined surgical table, with their head on the lower end. The uterus and ovaries were exteriorised by medioventral laparotomy, the number of CLs recorded, and puncture was performed at the base and the tip of each uterine horn. The flushing was performed by retrograde perfusion of 40 mL of Phosphate-Buffered Saline (PBS) + 2g/L Bovine Serum Albumin (BSA) + 50mg/L Kanamycin (Sigma-K1377, Sigma-Aldrich). The flushing medium was analysed and obtained embryos evaluated according to the criteria of International Embryo Transfer Society - IETS. The following structures were evaluated and counted: total number of collected forms, number of transferable and non-transferable embryos, and number of non-developed / retarded embryos. The rate of flushing success was calculated, as well as the percentage of fertilized oocytes, and vital and retarded embryos.

*Ultrasound exams.* The ovarian status of each animal was monitored by ultrasound examinations performed before and during superovulatory treatment, until ovulation occurred. The goats were placed in dorsal recumbent position and a 7.5 MHz lubricated linear array ultrasound probe was placed into the rectum (Sonovet 2000, Medison Co.). A concave rod was used to reinforce the probe, as it was attached to an insufficiently firm cable (GRIZELJ et al., 2013). The *lubricant containing local anaesthetic* was applied in a *rectal* ampulla 5 min before introducing the *rectal probe* (Lidocaine 2% diluted in ultrasound gel in ratio 1:10). The scanning of ovaries was done once a day before the start and during the superovulation protocol in order to count and measure the follicles  $\geq 2$ mm.

*Statistical analysis.* The animals that did not respond to superovulatory treatment (<5 CLs) were omitted from the statistical analysis concerning the results obtained after flushing the uterus. Statistical analysis was performed using the procedure program package SAS 9.1.3. Service Pack 4 (2002-2003 by SAS Institute Inc.). The probability of differences in superovulatory success between the analysed groups, and the difference

in the follicular dynamics was calculated using the SAS module PROC GLM when the fixed effects and PROC MIXED when random and fixed effects were analysed in the model. Pearson's correlation coefficient (PROC CORR) was used to identify the correlation coefficient of the number of CLs with the number of collected forms and the number of specific categories' follicles before the first FSH injection, with the number of collected forms and the number of transferable embryos. When the presumption of normal distribution of the analysed dependent variables and heterogeneity of variances was disturbed, the analysis was done using PROC GENMOD, or transformation of the dependent variable was used. The statistical significance was set at  $P < 0.05$ . Figures were made using the PROC GCHART module of SAS.

## Results

*Superovulatory response in goats.* Of the 30 goats in which superovulatory treatment was used a good response was obtained, which means at least 5 ovulations, in 25 animals (83.3%). One of them did not exhibit any behavioural signs of oestrus and hence was not mated. Three goats had an early luteal regression, and one did not ovulate at all.

*Uterine flushing efficiency rate.* A total of 219 ova/embryo forms were found in the flushed media, 311 CLs were counted on ovaries, and the average flushing efficiency rate (number of CL / number of collected cellular forms) was 73.2% ( $r = 0.80$ ;  $P < 0.0001$ ). The efficiency rate of the first and the second flushing was 74.1% and 71.8%, respectively.

Table 1. Distribution of evaluated collected forms (percentage of fertilized oocytes, degenerated and transferable embryos

No. of collected forms (oocytes and embryos)	No. of fertilized/collected forms	Degenerated embryos / collected forms	Transferable embryos / collected forms	Grade 1 and 2 / total embryos
219	81.7% (179/219)	14.6% (32/219)	61.1% (134/219)	74.9% (134/179)

At the start of the superovulatory treatment, 8 out of 25 goats had a dominant follicle present on their ovaries (size  $\geq 6$  mm); 7 of 13 from the control group (54%) and 1 of 12 from the day 0 group (8%). The follicular status at the beginning of the superovulatory treatment and superovulatory response for different protocols and animals with or without dominant follicle are shown in Table 2.

The average number of follicles within different follicular categories are shown in Figs 1 and 2, from the first FSH injection until ovulation occurred. The positive and statistically significant correlations between the number of second category follicles with the number of collected cellular forms and the number of transferable embryos were found ( $r = 0.62$  and  $r = 0.67$ ;  $P < 0.05$ ), respectively.

Table 2. Ovarian follicular status at the start of superovulation treatment and superovulation response. The control group was compared with the day 0 group, and the animals with a dominant follicle (DF) at time of the first FSH dose were compared with the animals without a dominant follicle (NO\_DF).

	Control group	Day 0 group	P value	DF	No DF	P value
Average size of the biggest follicle at the start of the protocol (mm)	5.46 ± 0.40	3.12 ± 0.40	<0.001	6.50 ± 0.31	3.32 ± 0.26	<0.001
Average size of all follicles present at the start of the protocol (mm)	3.14 ± 0.12	2.50 ± 0.07	<0.001	3.20 ± 0.20	2.66 ± 0.07	<0.07
Average size (mm) of all follicles at the start of the protocol, excluding the dominant follicles (≥ 6 mm)	2.88 ± 0.09	2.46 ± 0.07	<0.01	2.70 ± 0.11	2.66 ± 0.07	n.s.
Average size of all follicles 24 h after GnRH application (mm)	-	2.31 ± 0.12	-	-	-	-
Average number of CL	17.75 ± 1.88	10.89 ± 1.76	<0.05	12.63 ± 2.73	13.40 ± 2.30	n.s.
Average number of collected forms	12.67 ± 1.71	7.44 ± 0.95	<0.05	10.30 ± 1.81	10.70 ± 1.54	n.s.
Average number of transferable embryos	7.25 ± 1.51	5.22 ± 1.17	<0.05	3.85 ± 1.01	7.64 ± 1.31	<0.05
Average number of non-transferable embryos	5.00 ± 1.08	2.20 ± 1.00	n.s.	6.10 ± 1.60	2.59 ± 1.25	0.05
Average number of first grade embryos	5.92 ± 1.58	5.00 ± 1.31	n.s.	2.85 ± 0.98	7.00 ± 1.19	<0.05
Transferable embryos/total number of collected forms (%)	56.30 ± 7.63	69.87 ± 8.63	n.s.	40.84 ± 11.16	69.62 ± 6.47	<0.05

n.s. - not statistically significant

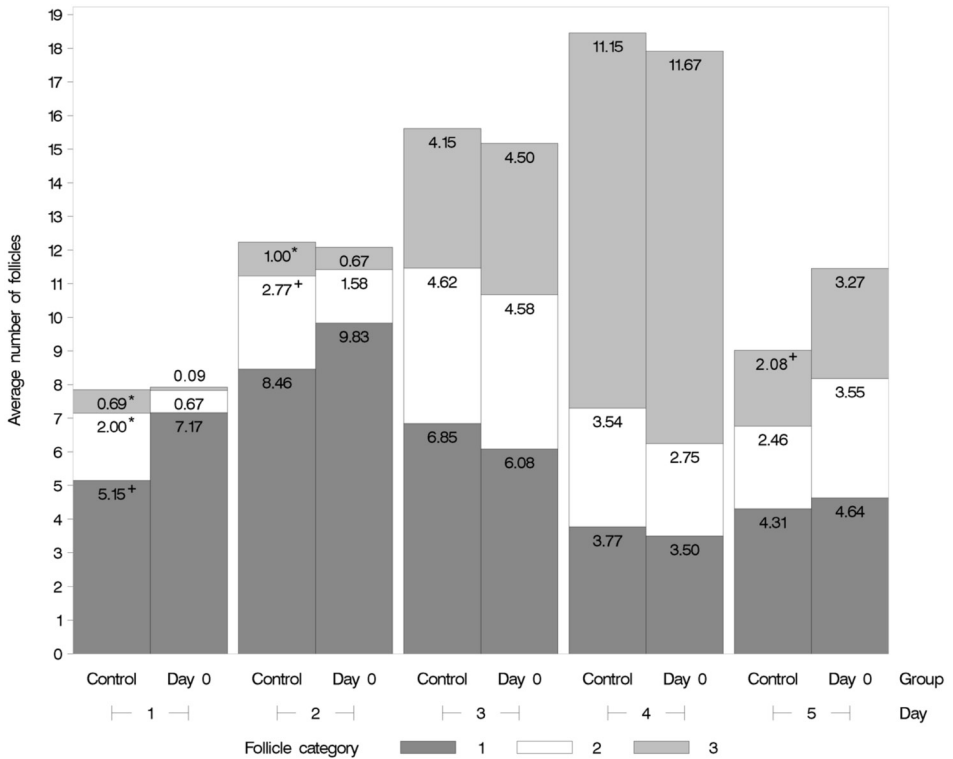


Fig. 1. Histogram showing a comparison of the average number of follicles within three follicular categories during the superovulation treatment between the control and the day 0 groups.

Follicular categories (subgroups): 1 ( $\varnothing$  2-3 mm), 2 ( $\varnothing$  3.5-4.5 mm), 3 ( $\varnothing \geq 5$  mm).

Day 1 (0 h, before the first FSH injection), 2 (before the third FSH injection; 24 h), 3 (before the fifth FSH injection; 48 h), 4 (at the start of estrus; 72-78 h), 5 (after the ovulation, 112-120 h).

\* Statistically significant difference in the number of follicles within the same follicle category between the control (C) and day 0 (D0) groups ( $P < 0.05$ ).

<sup>+</sup> Values that were close to statistically significant difference ( $P < 0.10$ ) in the number of follicles within the same follicle category between the control (C) and day 0 (D0) groups.

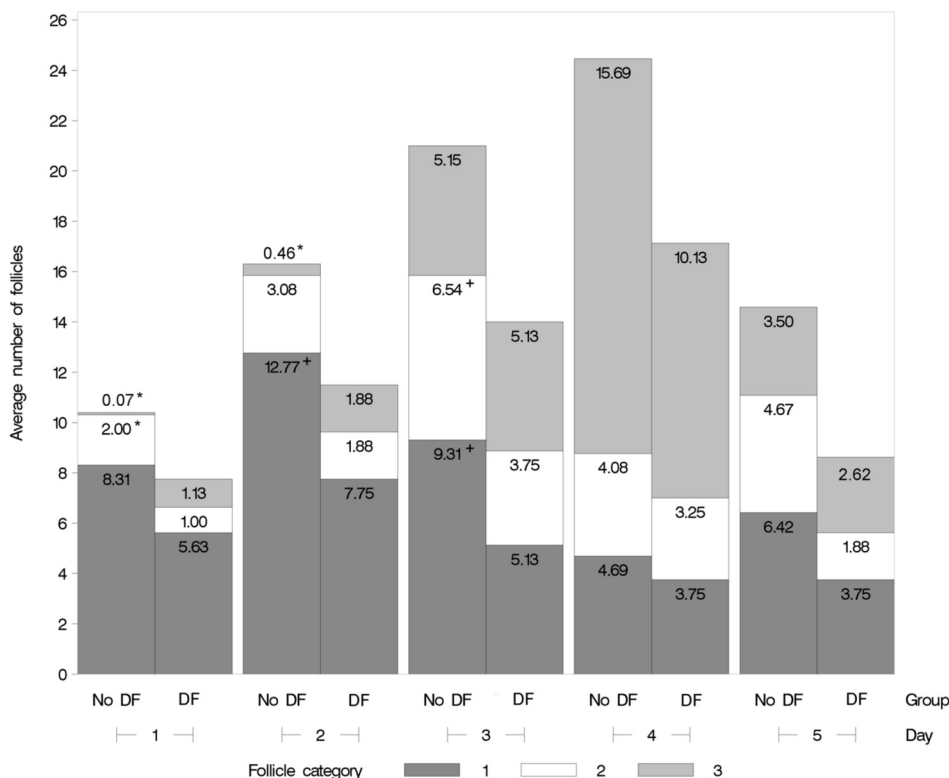


Fig. 2. Histogram showing comparison of the average number of follicles within three follicular categories during the superovulation treatment between animals with a dominant follicle present (DF) and those without a dominant follicle (NO\_DF) at the time of the first FSH injection.

Follicular categories (subgroups): **1** ( $\varnothing$  2-3 mm), **2** ( $\varnothing$  3.5-4.5 mm), **3** ( $\varnothing \geq 5$  mm).

Day **1** (0 h, before the first FSH injection), **2** (before the third FSH injection; 24 h), **3** (before the fifth FSH injection; 48 h), **4** (the start of oestrus; 72-78 h), **5** (after ovulation, 112-120 h).

\* A statistically significant difference in the number of follicles within the same follicle category between the group with a dominant follicle present (DF) and the group without a dominant follicle (NO\_DF) ( $P < 0.05$ ).

+ Values that were close to statistically significant difference ( $P < 0.10$ ) in the number of follicles within the same follicle category between the group with a dominant follicle present (DF) and group without a dominant follicle (NO\_DF).



## Discussion

The flushing success rate in this study is very close to the result obtained by SENLIS (1990) who had a laparotomic uterine flushing efficiency rate of 71%. In the current study, the success of the first flushing was slightly higher than the second one, with no statistically significant difference ( $P < 0.10$ ).

In the current study, the average number of CLs counted on the ovaries of all animals in both groups was  $14.8 \pm 1.5$ , which was consistent with previous research done by GONZALEZ-BULNES et al. (2003a), SENTHIL-KUMAR et al. (2003) and GREYLING et al. (2002), respectively. This number is one of the vital parameters used to evaluate the success of the superovulation treatment, and it depends on several factors, such as the source of gonadotropins, differences in protocol applied, the breed of the animals and the season in which the research was conducted (GARZA et al., 2015).

In this study a statistically significant difference was found in the total number of CLs between the control and day 0 groups of animals. The day 0 group had a significantly lower number of CLs, and this finding corresponds to the results obtained by a similar protocol in the study conducted by MENCHACA et al. (2007) and LEHLOENYA and GREYLING (2010).

The average number of collected forms for all the animals included in this study was slightly higher when compared with research done in other breeds of goats (PENDLETON et al., 1992; SELVARAJU et al., 2003; SENTHIL-KUMAR et al., 2003). This may indicate that Boer goats respond better to superovulatory treatment.

The average number of transferable embryos for all goats in this research was  $6.38 \pm 1.0$ , and the percentage of transferable embryos within the collected forms (embryo viability) was  $60.03 \pm 6.3\%$ , which is consistent with the results obtained by SENTHIL-KUMAR et al. (2003) and GONZALEZ-BULNES et al. (2003a).

The average number of non-transferable embryos in this research was almost five times higher than that in results obtained by SENTHIL-KUMAR et al. (2003). The same occurred with the number of non-fertilized oocytes, while the percentage of fertilization was similar to the results of other researchers (KHOBOSO, 2008; MENCHACA et al., 2007). Interestingly, the number of non-fertilized oocytes in the current study was two times lower than that obtained by KHOBOSO (2008).

When compared to the experimental group (day 0), the control group in the current study had a significantly higher number of collected forms and a higher number of transferable embryos which was close to statistically significant difference ( $P \leq 0.10$ ). On the other hand, concerning the number of collected forms and transferable embryos MENCHACA et al. (2007) in their research reported similar results obtained from the experimental group; however, their control group had a significantly smaller number of

collected forms and transferable embryos when compared to the results obtained from the control group in this study. The most likely reason for this discrepancy is that at the beginning of superovulation treatment a dominant follicle (measuring  $\geq 6$  mm) was observed in 85% of animals.

Moreover, animals with a dominant follicle at the beginning of the treatment showed asynchronous growth of the follicles during superovulation, and most of those did not ovulate, what probably caused the small number of transferable embryos obtained. However, it seems that the dominant follicle did not hamper the development of the other follicles during the treatment with FSH.

In the current study, there was no association between the number of follicles of each category, the time when the superovulation protocol started and the number of obtained transferable embryos. This fact suggests that, although the application of exogenous FSH may induce the development of the follicles to pre-ovulatory size, following ovulation and fertilization not all the oocytes have the capacity to become vital (transferable) embryos. Since there was no difference in the number of CLs and the number of collected forms between the animals with and those without a dominant follicle, we hypothesize that the most probable reason for the lower ovulation rate in the day 0 group could be a particular follicular population that was present at the beginning of the superovulation treatment. This is further supported by the results of similar studies in which the presence of a dominant follicle measuring  $\geq 6$  mm at the beginning of superovulatory treatment with multiple doses of FSH did not significantly influence the percentage of ovulation (GONZALEZ-BULNES et al., 2000, 2003a, 2003b).

Similar positive correlations between the number of follicles measuring 2 to 6 mm and the number of CLs, as well as the number of follicles measuring 4 to 6 mm and the number of transferable embryos, were found by GONZALEZ-BULNES et al. (2003a). According to DE SMEDT et al. (1994) oocytes are capable of reaching maturity only within follicles measuring more than 3 mm in diameter. Oocytes that come from smaller follicles are either hard to fertilize or develop following fertilization into retarded or non-transferable embryos. Likewise, in our study, the group of goats without a dominant follicle had a significantly higher percentage of transferable embryos, and particularly a higher number of first class embryos. On the other hand, the group of goats with a dominant follicle had a greater number of non-transferable embryos, and the difference was close to statistically significant ( $P = 0.07$ ). The average diameter of the follicles in the day 0 group on the first day of the experiment was significantly lower than in the control group. Such a low average follicular diameter on the first day of the experiment could be the result of the particularities of the applied protocol, i.e. the fixed time when the superovulation protocols began. The day 0 protocol began 24 h following induced ovulation, i.e. 48 h after GnRH application. For the majority of goats, twenty-four hours

after induced ovulation was obviously an insufficient length of time for development of follicles larger than 3 mm in diameter. The slower follicular growth after ovulation could indicate that Boer goats may be prone to have a smaller number of follicular waves during the sexual cycle, while most authors indicate four follicular waves as an average in the sexual cycle of goats (GINTHER and KOT, 1994; DE CASTRO et al., 1999; SCHWARZ and WIERZCHOS, 2000; MENCHACA and RUBIANES, 2002). Another reason for the slower follicular growth could be application of the GnRH analogue, given 48 h before the start of the superovulation treatment in order to synchronize ovulation. Likewise, less successful flushing and a smaller number of transferable embryos obtained were recorded in a study where a GnRH analogue was given as part of the superovulation protocol (KHOBOSO, 2008). The author suggests that the reason for the failure of the superovulation treatment could be the low levels of LH hormone that impeded maturation of the oocytes. These results are contradictory to a previous research where a higher number of transferable embryos were obtained (COGNIE et al., 2003).

### **Conclusions**

The modified day 0 protocol significantly reduced the number of animals with a dominant follicle present before the start of superovulation. It also reduced the number of follicles measuring 3 to 5.5 mm. Goats that had a dominant follicle present at the time when the superovulation procedure started had fewer second category follicles at the time when the first FSH dose was injected, and fewer transferable embryos.

The animals with dominant follicles had uneven follicular growth due to atresia and disturbed follicular recruitment, and they had a large number of non-transferable embryos. In Boer goats, the number of follicles of a certain size before the start of the superovulation treatment has an effect as important as the effect of dominance.

The application of FSH hormone when performing the modified (day 0) protocol should start at the time when a suitable number of follicles belonging to the second category are present on the ovaries.

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**SAŽETAK**

Cilj ovog istraživanja bio je istražiti utjecaj jajnika na uspjeh dvaju superovulacijskih protokola u koza. Petnaest je koza u istraživanju korišteno dva puta u ukriženom dizajnu; prvi puta u kontrolnoj skupini standardnoga protokola, a nakon toga u modificiranom „dan 0” protokolu gdje nije bilo prisutnog dominantnog folikula, a folikularna kohorta je homogena. Sve životinje sinkronizirane su vaginalnim spužvicama te superovulirane sa 6 primjena padajućih doza pFSH. Iako je „dan 0” protokol znakovito smanjio prisutnost dominantnog folikula prije početka superovulacijskog postupka, protokol nije poboljšao uspjeh superovulacije. Pedeset i četiri posto koza u kontrolnoj skupini imalo je dominantan folikul koji je ispoljio negativan učinak na broj transferabilnih zametaka. Ostalih 46 % koza iz iste skupine imalo je vrlo dobar superovulacijski odgovor što je popravilo ukupni odgovor kontrolne skupine u odnosu na „dan 0” skupinu. Drugi razlog za slabiji uspjeh superovulacije u „dan 0” protokolu je manji broj folikula druge i treće kategorije (promjera 3,5 - 5,5 mm) na početku superovulacijskog protokola koji su u pozitivnoj korelaciji s brojem transferabilnih zametaka ( $r=0,67$ ;  $P<0,05$ ). Za uspjeh superovulacijskog protokola u burskih koza osim učinka dominacije od iznimnog su utjecaja brojnost folikula određene veličine na početku superovulacijskog protokola. Primjena FSH hormona pri izvođenju „dan 0” protokola treba započeti u vrijeme kada na jajnicima postoji odgovarajući broj folikula druge kategorije.

**Ključne riječi:** koza, superovulacija, dominantni folikul, folikularna populacija, zametak, superovulacijski uspjeh

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