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Embryo Flushing in Cows under Various Superovulation Schemes

Maksym Salizhenko*, Oleksandr Valchuk, Vitalii Kovpak, Serhii Derkach, Yurii Masalovych

National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine

Abstract. The use of biotechnological reproduction methods is a relevant issue since the embryo transfer, obtained after stimulating superovulation, can accelerate reproduction and improve the number of cattle. The purpose of the study was to evaluate the effectiveness of various schemes for stimulating superovulation in cows of the Ukrainian black-pock dairy breed. Therewith, the study analysed the ovarian response to the drug "FSH-Super" under different introduction schemes: Step-up (gradual increase in the dose) and Step-down (gradual dose reduction) and recorded the number of embryos suitable for transplantation. Donor cows were administered the drug "Estrofan" to synchronise the sexual cycle. After 7 days, the drug "Ovarelin" was injected, and after another 7 days, the injection of the drug "Estrofan" was repeated in the same dose. Stimulation of superovulation began on the 10th day of the sexual cycle with the drug "FSH-super" in the form of eight gradually increasing (Step-up) and gradually decreasing (Step-down) doses within 4 days. Artificial insemination was performed 12 and 24 hours after the start of oestrus. During the study, it was discovered that in the group of cows with gradual dose reduction of the drug "FSH-Super", 83.3% reacted with superovulation, and in the group with the gradual increase – only 71.4%. Therewith, the number of yellow bodies on two ovaries in a donor cow averages 15.6 and 9.2, respectively. An average of 12.4 and 7.8 embryos (Step-down and Step-up) were obtained from the donor, of which 7.8 and 4.2 are suitable for transplantation, respectively. However, in the group of cows with gradually decreasing doses, a higher number of embryos unsuitable for transplantation was obtained – 4.6 and unfertilised oocytes – 2.6, compared with the group of donors with gradually increasing doses, where these indicators are 3.6 and 1.0, respectively. Thus, the use of the drug "FSH-super" to donor cows according to the step-down introduction scheme allows getting more embryos suitable for transplantation. This will allow managing the biotechnological aspects of cattle reproduction and effectively and in a controlled manner accelerate the breeding process in farms of various forms of ownership, fixing the desired genotype in the herd

Keywords: FSH-Super, cattle, follicle, Step-up, Step-down

Introduction

According to the forecasts of researchers, by the middle of the 21st century, the world's population will grow to 9.5 billion people, which will require an increase in the global production of food products, including animal origin, and as a result, will cause a substantial number of problems associated with their production [1; 2].

The use of modern biotechnological methods of reproduction of cattle, and careful selection and culling of animals, considering their genetic potential, can contribute to solving the problem of increasing the production of meat and dairy products [2], because the reproductive ability of cows determines the economic efficiency of cattle breeding. A substantial obstacle to the rapid increase in the number of highly productive cows is the long period of pregnancy and the ability to bear only one embryo. Despite the fact that only a small number of oocytes are sold during economic use,

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*Corresponding author

cows have a generative potential of hundreds of thousands of oocytes. That is why the use of biotechnological reproduction methods is quite important and always relevant. Transplant embryos obtained after stimulation of superovulation, can accelerate reproduction and improve the number of cattle [3].

After the first successful mammalian embryo transfer in 1890, approximately 60 years passed before progress in basic bovine embryo transfer technology was reported [4]. The commercialisation of bovine embryo transfer began in North America in the early 1970s [5]. However, the specialists of that time used surgical methods of both flushing and embryo transfer. It wasn't until 1987 that Craig Smith introduced the concepts of multiple ovulation (superovulation) and embryo transplantation (MOET) at the University of Guelph (Canada). It demonstrated how well-designed MOET programmes can lead to increased selection intensity and shorter generation intervals, leading to improved genetic stock.

Currently, there are dozens, if not hundreds, of commercial enterprises worldwide that use biotechnological methods, which include superovulation, embryo extraction from the donor's uterine horns, embryo transfer, cryopreservation, and in vitro fertilisation [7; 8]. Bovine embryo transfer is a big international business. Every year, over five hundred thousand bovine embryos are produced worldwide using superovulation stimulation techniques [7]. According to the International Society for Embryo Transplantation (IETS), the number of embryos that were transplanted and obtained by the method *in vivo* for one donor cow in the world is 6.7 [9]. For its part, the American Embryo Transplantation Association [10] states that the number of transplanted embryos obtained by the method *in vivo* per donor cow is 6.6 – for meat and 5.7 – for dairy breeds of cattle.

The procedures used in cattle breeding biotechnology have already been defined, but there are opportunities to improve them. For example, superovulation plays an important role in embryo transplant programmes. Its purpose is to ovulate a large number of follicles and a high yield of good quality embryos suitable for transplantation. One of the most problematic aspects of this stage is the variable response of the donor animal to superovulation stimulation and, as a result, unstable superovulation results [11]. Despite much attention paid to this issue, little progress has been made over the past 40 years. Therefore, researchers around the world continue to search for optimisation of existing schemes for stimulating the superovulation of cattle.

Given the above, optimisation of superovulation stimulation schemes in cattle is quite relevant, so the purpose of this study was to compare the effectiveness of two approaches to superovulation stimulation Step-up (gradual increase) and Step-down (gradual decrease) in the dose of exogenous follicle-stimulating hormone (FSH).

The purpose of the study was to evaluate the effectiveness of various schemes for stimulating superovulation in cows of the Ukrainian black-pock dairy breed.

Literature Review

The basic principle of superovulation and transfer of embryos are the production of embryos from a donor animal with a higher genetic value, after which the embryos are transferred to recipient animals with a lower genetic value. The method is aimed at increasing the number of offspring from the donor, while slowing the spread of recipient genes among the herd [5].

International embryo trade is a growing business, and embryos are also considered a safe way to exchange genetic material between countries. Since the risk of disease transmission is negligible for most pathogens, provided that the recommendations of the International Society for Embryo Transplantation are followed [12]. The main stages of superovulation and embryo transfer are: stimulation of ovarian follicle growth with gonadotropins, induction of oestrus with luteolytic drugs, insemination and flushing of embryos on the 7th day after the first insemination, and transfer of fresh embryos to synchronised recipients or freezing of embryos [5].

Stimulation of superovulation usually begins in the middle of the luteal phase of the oestrous cycle – from 8 to 12 days after oestrus. The physiological principle of this approach is based on follicular waves, of which there are usually two or three during the oestrous cycle, the second occurs on about 10 days, in most cycles. The goal is to target exogenous follicle-stimulating hormone (FSH) to new follicles that respond to gonadotropin, avoiding the effect of the dominant follicle on them [5].

It is possible to identify donor cows with as many new follicles as possible by determining the level of antimulerer hormone (AMH) [13]. This allows assessing the ovarian reserve and selecting cows with a potentially better response to stimulation of superovulations.

As for the groups of drugs for superovulation, the most widely used drugs are: pituitary FSH and pregnant mare serum gonadotropin (PMSG) [14; 15].

Pregnant mare serum gonadotropin (PMSG) is a serum, complex glycoprotein, that contains luteinising hormone (LH) and FSH [16]. Substantial variability in the results of stimulation of superovulation with PMSG drugs is due to the not optimal ratio of FSH and LH fractions, and their insufficient concentration [17]. In addition, PMSG drugs cause cystic changes in the ovaries and stimulate the production of antibodies, which reduces the possibility of repeated use of donor cows [18].

Therefore, now purified preparations of pituitary origin are increasingly used to induce superovulation, with a ratio of FSH to LH, as one to one or one to two, respectively [19], with the presence of sialic acid in the preparation, which ensures its active effect. Notably, that pituitary drugs, depending on the degree of their purification, have a different pharmacologic half-life. Thus, this indicator is for "FOLLTROPIN" (the main active substance – FSH extracted from the pituitary gland of pigs, 700 IU in a bottle) and "Pluset" (the main active substance – FSH and LH, extracted from the pituitary glands of pigs, 500 IU in a bottle) is up to 6 hours [11], while for "FSH-Super" (the main active substance is FSH extracted from the pituitary glands of pigs, 1000 IU in a bottle) – up to two days. Due to the relatively short pharmacologic half-life of available FSH preparations, two daily injections (with an interval of 12 hours) are necessary to maintain an equilibrium level of FSH during ovarian stimulation [20].

Currently, there are two approaches to stimulating superovulation using follicle-stimulating hormone in the world. Thus, in North America, a high initial dose of FSH (Step-down) is usually used with a gradual decrease in it, while in Europe – a low (Step-up) with a subsequent increase in the level of the hormone. [21].

Materials and Methods

Experiments on cows were conducted based on the Faculty of Veterinary Medicine of the National University of Bioresources and nature management of Ukraine and LLC "Golden Meadows" in compliance with the requirements of the European Convention for the Protection of Vertebrate Animals [22] used for Experimental and Other Scientific Purposes of 1986, and the Law of Ukraine "On the Protection of Animals from Cruel Treatment" of 02/21/2006 No. 3447-IV as amended on 04.08.2017 [23].

The study was conducted in the period from December 2019 to March 2020 in LLC "Zoloti Luky" village of Pariivka, Illinetskyi district, Vinnytsia region, on 13 clinically healthy cows of the Ukrainian black-pock dairy breed, aged from 4 to 8 years with a body weight of 600-650 kg, on the 45th-120th day of lactation, with a pre-lactation yield of 7-8 thousand kg of milk, with a fat content of over 3.6%.

Animals with a plasma concentration of antimulerer hormone in the range of 0.1-0.2 ng/cm^3 were selected for the experiment. Blood for this study was taken before the start of superovulation stimulation. Blood was taken from the tail vein into vacuum tubes with a coagulation activator. Then the blood tubes were settled at room temperature (18-20^{o}C) to separate the serum from the clot. Undiluted blood serum (20 μL) was used for analysis. The concentration of AMH was determined by enzyme-linked immunosorbent analysis (ELIA) using the AccuBind® AMH test kit (Monobind – Los Angeles, USA).

All experimental animals were divided into two groups according to the analogue principle (n = 13). In the first group, the ovarian response (number of yellow bodies) to the drug "FSH-Super" (the main active substance – FSH extracted from the pituitary glands of pigs, 1000 IU in a bottle, manufacturer "Agrobiomed", RF) was investigated and the number of embryos suitable for transfer was determined for using the superovulation stimulation scheme Step-up (gradual increase in the dose of the drug under study), in the second group – for using the superovulation stimulation scheme Step-down (gradual decrease in the dose of the drug under study). All experimental animals were subjected to synchronisation of the sexual cycle, regardless of the initial stage.

Donor cows were given 2 cm^3 of the "Estrofan" drug intramuscularly (Bioveta Czech Republic) to synchronise the sexual cycle. After 7 Days, 2 cm^3 of the drug "Ovarelin" (Ceva Sante Animale, France) were injected intramuscularly, and after another 7 days repeated the injection of the drug "Estrofan" in the same dose.

Cows were subjected to two-time artificial insemination by the recto-cervical method 12 and 24 hours after the start of oestrus. Using frozen-thawed sperm in straws with a volume of 0.25 cm^3 with a sperm concentration of 50-60 million. FSH-Super (Agrobiomed, RF) was used to compare different superovulation schemes (Table 1).

Table 1. Schemes for stimulating superovulation of donor cows with FSH-Super

Sexual cycle, day	Input time							
	Experimental group of donor cows no. 1				Experimental group of donor cows no. 2			
	8.00 h		20.00 h		8.00 h		20.00 h	
	FSH-Super, IU	Estrofan, cm^3	FSH-Super, IU	Estrofan, cm^3	FSH-Super, IU	Estrofan, cm^3	FSH-Super, IU	Estrofan, cm^3
0	*Oestrus*							
10	100	–	100	–	250	–	250	–
11	150	–	150	–	200	–	200	–
12	200	4	200	–	150	4	150	–
13	250	–	250	–	100	–	100	-
14 (1)	–		Oestrus		–		Oestrus	
	Insemination							
7	Embryo flushing							

Superovulation stimulation was performed for four days and started after synchronisation on the 10th day of the sexual cycle. Notably, that in the investigated schemes, the total dose of the hormone was identical and amounted to 1400 IU.

Before embryo flushing, donor cows underwent ovarian sonography to record the number of yellow bodies using the KX5200 ultrasound device (Kaixin, China).

Embryo flushing from each horn was performed alternately through a 2-channel silicone catheter CH20 with a Foley-type connection (Minitube, Germany) and the EmSafe filter system for flushing cattle embryos (Minitube, Germany) using a phosphate buffer solution (Sigma, USA) with the addition of 0.05 g/dm^3 of bovine serum albumin (Sigma, USA), and 12 μg/cm^3 of gentamicin (Arterium, Ukraine). The medium was used at the rate of 500 cm^3/uterine horn. Flushing was conducted by gravity method. At the end of flushing, the filter was cleaned using 50 cm^3 of Eagle environment (modified Dulbecco environment) using a sterile plastic syringe with a capacity of 50 cm^3 (Minitube, Germany). After the procedure, each animal was injected with 3 cm^3 of the drug "Estrofan" ("Bioveta", Czech Republic) for lysis of yellow bodies.

Using the SZM-45B stereomicroscope (Spectro Lab, Ukraine), embryos were searched and evaluated by quality and stage of development in accordance with the recommendations of the International Society for Embryo Transplantation [24].

Results and Discussion

In this experiment, 7 cows were selected for experimental group No. 1, and 6 cows with an average AMH level of 0.15 ng/cm^3 were selected for experimental group No. 2 (Table 2).

Table 2. AMH level and number of yellow bodies in cows of experimental groups

Experimental group of donor cows No. 1				Experimental group of donor cows No. 2			
No.	No. of cow	AMH, ng/cm^3	Number of yellow bodies	No.	No. of cow	AMH, ng/cm^3	Number of yellow bodies
1	6768	0.14	8	1	8611	0.18	17
2	8452	0.19	12	2	8447	0.19	15
3	8527	0.18	9	3	8492	0.15	14
4	7264	0.16	8	4	6893	0.12	13
5	8531	0.17	9	5	8508	0.20	19
6	7114	0.13	-	6	7140	0.10	-
7	6979	0.12	-	-	-	-	-
M ± m	-	0.15 ± 0.01	9.20 ± 0.73	-	-	0.15 ± 0.01	15.60 ± 1.07

Considering the same average level of AMH in the experimental groups, it can be argued that they are balanced and have the same ovarian reserve. The level of AMH among cows in the study groups ranged from 0.10 to 0.20 ng/cm^3.

In experimental group No. 1, out of seven cows exposed to superovulation stimulation, only five responded. The two cows that did not respond had the lowest AMH levels among the group – 0.12 ng/cm^3 and 0.13 ng/cm^3, respectively. A similar result was observed in experimental group No. 2, where only one of the six cows with the lowest level of AMH – 0.10 ng/cm^3, did not react. Depending on the scheme of FSH introduction, different results were obtained, despite the fact that both experimental groups responded to superovulation stimulation of 5 cows, and the average group level of AMH is identical.

The effectiveness of the effect of "FSH-super" on the manifestation of superovulation under different introduction schemes was determined and it was established that in experimental group No. 2 cows that reacted with superovulation were 5 out of 6 heads, that is, 83.30%, and in experimental group No. 1 – 5 out of 7 heads, 71.40%, respectively.

Before the embryo flushing procedure, an ultrasound examination of the ovaries of donor cows was performed to determine the number of yellow bodies (Fig. 1). Obtained and evaluated embryos (Figure 2) in accordance with the recommendations of the International Society for Embryo Transplantation. [24]. The obtained data were processed, and the results are presented in Table 3.

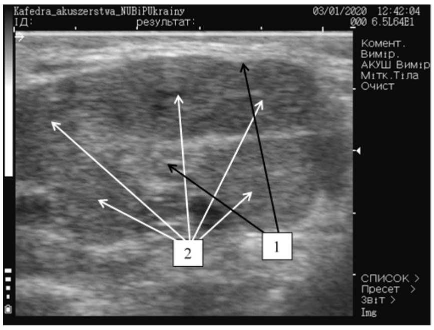

Figure 1. Ultrasound examination of the ovary of a donor cow

Note: 1) ovarian parenchyma, 2) corpus luteum

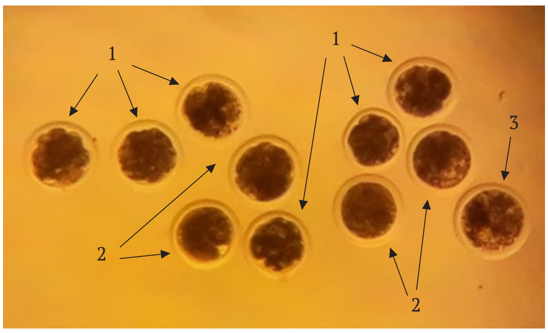

Figure 2. Cattle embryos at different stages of development obtained by flushing after stimulation of superovulation with FSH-super

Note: 1) early morula, 2) late morula, 3) early blastocyst. Native drug, ×100

Table 3. Impact effectiveness of "FSH-super" on the manifestation of superovulation in donor cows under different introduction schemes, M ± m

Indicator	Experimental group of donor cows No. 1	Experimental group of donor cows No. 2
Number of animals, n	7	6
Donor cows that responded with superovulation, n (%)	5 (71.40)	5 (83.30)
Number of yellow bodies in 1 donor	9.20 ± 0.73	15.60 ± 1.07*
Number of embryos received from 1 donor	7.80 ± 0.82	12.40 ± 1.42*
Number of embryos suitable for transplantation, %	4.20 ± 0.58 (53.84)	7.80 ± 0.73 (62.90)*
Number of degenerated embryos	3.60 ± 0.24	4.60 ± 0.24
Number of oocytes	1.00 ± 0.32	2.60 ± 0.40
Fertilisation rate, %	84.78	79.40

Note: *P < 0.05, probably compared to the experimental group of donor cows No. 1

Based on the results obtained, presented in Table 3, it follows that the average number of yellow bodies on the ovaries of donor cows in experimental group No. 2 is 1.69 times (P < 0.05) higher than in donor cows in experimental group No. 1. Therewith, the number of embryos obtained from 1 donor of cows of experimental group No. 2 is 1.59 times (P < 0.05) higher than that of donor cows of experimental group No. 1. Thus, the quantity of suitable for transplantation embryos in donor cows of experimental group No. 2 is 1.86 times larger than in donor cows of experimental group No. 1.

However, there was no statistically substantial difference in the number of degenerated embryos in donor cows of experimental group No. 2 compared to experimental group No. 1, although this number was higher. Therewith, the number of oocytes in donor cows of experimental group No. 1 is less compared to donor cows of experimental group No. 2, and fertilisation is higher by 5.38%.

There is a direct pattern between low plasma AMH levels and low reproductive performance of cows [25], which was confirmed by this study because cows with the lowest AMH values did not respond to superovulation stimulation. According to data of B. M. Guerreiro *et al.* [13], cows with high AMH levels have more visible aspirated follicles, respectively, more oocyte-cumulus complexes and subsequent embryos.

Thus, in this study, the number of embryos obtained in one flushing was 10.1, similar to the data obtained by Chebel *et al.* – 10.9 [26]. However, the number of yellow bodies was twice as low – 12.4 compared to the data of Garcia Guerra *et al.* – 24.0 [19].

According to V. Chankitisakul *et al.* [17], the number of yellow bodies corresponded to a value of 11.4, and the suitability for embryo transplantation was 61.24%. For comparison, in experimental group No. 2 (Step-down) of the conducted study, the results obtained were 15.60 and 62.90%, respectively. Therewith, in experimental group No. 1 (Step-up), the number of yellow bodies was 9.20, and the suitability for embryo transplantation was 53.84%.

According to M. Drillich *et al.* [27], the number of embryos suitable for transplantation was 7.8, and according to this study – 6.0. However, according to the data of L. Vieira *et al.*, the number of embryos suitable for transplantation corresponded to a value of 2.4 [28]. This is due to the difference in superovulation stimulation patterns. Notably, the level of gonadotropic hormone in the blood of a donor cow, its individual characteristics, and the feeding factor can affect the number of embryos suitable for transplantation obtained during one flushing. [29].

The results obtained indicate an active superovulatory response, which is confirmed by data of H. Kohram and M. Poorhamdollah [30], who claim to have obtained 4 to 6 transplant-eligible embryos after superovulation in lactating cows. However, Peippo *et al.* [31] obtained 2.2 degenerated embryos in one flushing, and in this study, the number is 4.1.

Conclusions

Determination of serum antimulerer hormone levels can serve as a marker for selecting donor cows. Ultimately, animals with a deficient level of it show a low result or its absence. With an identical dose of the drug "FSH-super" (1400 IU) to stimulate superovulation, the use of the Step-down scheme allows getting an average of 7.80 (62.90%) embryos suitable for transplantation from donor cows, which is 3.60 (9.10%) more compared to the Step-up scheme, for the use of which to donor animals, this Figure is 4.20 (53.80%). The potential of a sufficient or substantial ovarian reserve may not be identified due to an unsuccessful selection of the superovulation stimulation scheme.

Promising areas are the investigation of the correlation between the level of antimulerer hormone in the blood serum of cows and the number of follicles that respond to superovulation stimulation. It is also necessary to determine the correlation between the level of antimulerer hormone in the blood serum of cows, ovarian reserve, and reproductive longevity. In addition, it remains a priority to examine the effect of the dose and method of introduction of exogenous follicle-stimulating and luteinising hormones on the superovulatory response and the yield of embryos suitable for transplantation.

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Вимивання ембріонів у корів за різних схем суперовуляції

Максим Ігорович Саліженко, Олександр Анатолійович Вальчук, Віталій Васильович Ковпак, Сергій Степанович Деркач, Юрій Степанович Масалович

Національний університет біоресурсів і природокористування
03041, вул. Героїв Оборони, 15, м. Київ, Україна

Анотація. Використання біотехнологічних методів відтворення є актуальним питанням, оскільки трансплантація ембріонів, отриманих після стимулювання суперовуляції, здатна прискорити відтворення та поліпшити поголів’я великої рогатої худоби. Метою роботи було оцінити ефективність різних схем стимуляції суперовуляції в корів української чорно-рябої молочної породи. При цьому, в роботі вивчали реакцію яєчників на препарат «ФСГ-супер» за різних схем введення Step-up (поступове підвищення дози) та Step-down (поступове зменшення дози) і реєстрували кількість ембріонів придатних до трансплантації. Для синхронізації статевого циклу коровам-донорам вводили препарат «Естрофан». Через 7 діб ін’єктували препарат «Оварелін», а ще через 7 діб повторювали ін’єкцію препарату «Естрофан» у тій же дозі. Стимуляцію суперовуляції розпочинали на 10 добу статевого циклу за допомогою препарату «ФСГ-супер» у вигляді восьми поступово зростаючих (Step-up) і поступово спадаючих (Step-down) доз упродовж 4 діб. Штучне осіменіння проводили через 12 і 24 год від початку охоти. Під час дослідження було встановлено, що в групі корів з поступовим зменшенням доз препарату «ФСГ-супер» зреагували суперовуляцією 83,3 %, а в групі з поступовим їх підвищенням лише 71,4 %. Водночас кількість жовтих тіл на двох яєчниках у корови-донора в середньому становить 15,6 і 9,2, відповідно. Від донора у середньому отримано 12,4 і 7,8 ембріонів (Step-down та Step-up), із них придатних до трансплантації 7,8 і 4,2, відповідно. Проте, в групі корів з поступово спадаючими дозами отримано більшу кількість непридатних до трансплантації ембріонів – 4,6 і незапліднених яйцеклітин – 2,6, порівняно з групою донорів, яким застосовували поступове підвищення доз, де ці показники становлять 3,6 і 1,0, відповідно. Таким чином, використання коровам-донорам препарату «ФСГ-супер» за схемою введення Step-down дає можливість отримати більшу кількість придатних до трансплантації ембріонів. Це дозволить управляти біотехнологічними аспектами відтворення великої рогатої худоби та ефективно й контрольовано пришвидшити селекційний процес у господарствах різних форм власності, закріплюючи у стаді бажаний генотип

Ключові слова: ФСГ-супер, велика рогата худоба, фолікул, Step-up, Step-down