Romanian Biotechnological Letters *Vol. 23, No. 5, 2018*

ORIGINAL PAPER

Follicle deviation and oocyte recovering by ovum pick-up from endangered Romanian Grey cows

DOI:10.26327/RBL2018.202

Received for publication, November 10, 2014
Accepted, June 27, 2015

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Abstract

Romanian Grey is an endangered, ancient breed (part of the Podolian group) which was recently included in a governmental program of genetic conservation. This study aimed to test the suitability of the rustic Romanian Grey to assisted reproductive techniques such as ovum pick-up (OPU) and to describe some details of its sexual cycle that could be useful when applying such procedures.

The interval from ovulation to deviation, the diameter of the dominant follicle (DF) and large subordinate follicle (SF) at deviation was determined in 10 Romanian Grey cows. The same cows were then used to perform OPU, two times (OPU 1, 2) at 3 weeks interval, on days 6 and 13-14 after estrus detection. The follicular wave was initiated by induced estrus and by puncturing the dominant follicle and all other follicles sized ≥3 mm at days 6 and 13-14 of the cycle. No hormones were used in OPU 1, while equine chorionic gonadotrophin was used on days 2 and 9 of cycle for follicular growth stimulation in OPU 2. The follicle deviation occurred at 2 ± 0.3 d (mean ± S.E.M.) after ovulation, and the diameters for DF and SF at deviation were 8.1 ± 0.2 mm and 7 ± 0.3 mm. The size of the dominant follicle at ovulation was 12.2 ± 0.1 mm. Mean number of aspirated follicles/cow/session was 6.2 ± 1.5 and 13.1 ± 2 in OPU 1 and OPU 2 respectively, while mean number of oocytes/cow/session was 4.20 ± 0.2 and 11.36 ± 0.3 in OPU 1 and OPU 2 respectively.

Our study shows that biotechnologies such as OPU can be applied successfully in Grey cattle, with or without hormonal stimulation, although superior results are obtained when ovarian stimulation is used. Further work will continue with in vitro fertilization of the oocytes obtained through OPU in order to obtain transferable embryos.

Keywords: Romanian Grey, OPU, follicle deviation, endangered cattle.

1. Introduction

Over the past 15 years, 300 out of 6000 breeds of all farm animal species identified by Food and Agriculture Organization of the United Nations (FAO) have gone extinct (1). The World Watch List for Domestic Animal Diversity (DAD) indicates that 1350 farm animal breeds currently face extinction. Romanian Grey, Hungarian Grey, Maremmana and Turkish Grey belong to the same Podolian group of cattle and show a similar external conformation. They recently underwent a similar demographic reduction, mainly due to low productivity compared to modern breeds. Podolian cattle include an ancient group of breeds, considered

to be straight descendants from the auroch (*Bos primigenius*) (2). Their name refers to a common ancestral origin in Podolia (the modern western Ukraine). However, place of origin and timing of spread out of the source area are both debated (3). Podolian breeds are present in various European areas, and many of them are seriously endangered of extinction. As of 2003, FAO (DAD-IS) included Romanian Grey on the list of endangered cattle (4).

The National Agency for Amelioration and Reproduction in Animal Husbandry (ANARZ – Romania) estimated in 2012 that small populations (n = 150) of pure and half-breed Romanian Grey variety could still be found, with a different absorption level, isolated, in the North-East of Romania and the Danube Delta. Despite its low productivity, the Romanian Grey is a valuable reservoir of genes with high importance for husbandry, due to characteristics such as high longevity, adaptability, hardiness, resistance to diseases and extreme temperatures, high fat and protein content of milk (5). In this context it is necessary to preserve its genetic resources by increasing, reproducing and improving its population and for this reason it was included in a governmental program of genetic conservation (6) which implies reproductive isolation, reduction and/or removal from the sphere of influence of the factors that may modify the gene frequency so that they can remain unchanged.

The transvaginal ultrasound-guided follicle aspiration (Ovum Pick-Up or OPU) represents a non-invasive procedure for recovering oocytes from antral follicles in live animals. OPU technique was adopted in cattle from human medicine by a Dutch team (7) who demonstrated that the repeated oocyte collection by OPU could be performed without risks for health and the reproductive activity. This method can be applied successfully irrespective of the reproductive status of the donor, and can yield more transferable embryos per donor on a monthly basis (8). It could be a good alternative to increase the probability of preservation of endangered Romanian Grey. The combination of OPU with *in vitro* embryo production (IVP) using either frozen semen from 1970 (bull Sura Fort II from ANARZ Bucharest) or semen from present bulls and the transfer or cryopreservation of obtained embryos would offer the opportunity to accelerate the recovery of this breed.

The aim of this study was to test the suitability of the rustic and endangered breed of Romanian Grey for assisted reproductive technologies such as OPU and to describe some important details of its sexual cycle that could be useful when applying such procedures.

2. Material and methods

2.1. Experimental animals

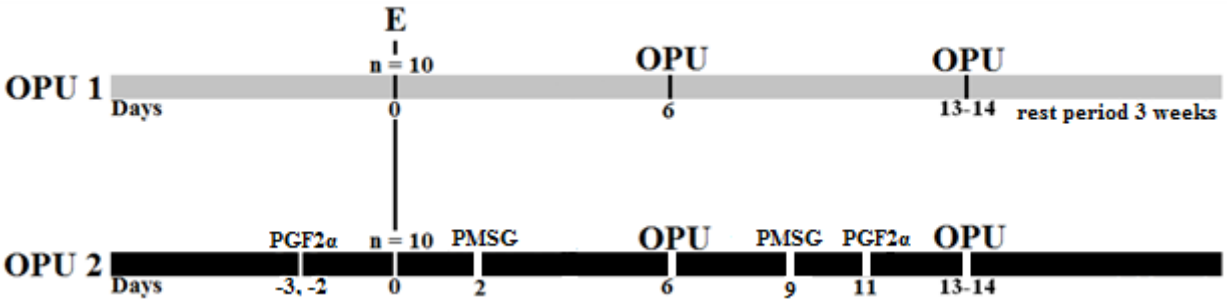
This study was performed at the Research and Development Station for Cattle Breeding Dancu-Iasi, Romania, which owns a population of Romanian Grey cows (Moldavian variety) included in a governmental program of genetic conservation (6). This population contains 59 females with ages between 6 months and 21 years and two males aged between 2-3 years. The animals used within study (n = 10) were multiparous, free from reproductive abnormalities, with ages between 4-5 years, body weight ranging from 420-480 kg and body condition scores of 4-4.5 (on a 0 to 5 point scale); they were kept in free stall system with a diet of 70% corn silage and 30% alfalfa hay.

The OPU technique was applied in two steps on the 10 cows: without any hormonal stimulation (OPU 1) and with hormonal stimulation program (OPU 2). The interval between OPU 1 and OPU 2 was a period of 3 weeks (or at least one estrus cycle).

2.2. Experimental design and stimulation protocol

The cows used for the experiment (n = 10) were subjected to four sequential sessions of OPU, for a total of 40 transvaginal aspiration procedures. First two aspirations (OPU 1) were applied on days 6 and 13-14 after detectable estrus, without any hormonal treatment. The third

and the fourth aspiration (OPU 2) were applied on days 6 and 13-14 after induced estrus following hormonal stimulation. The cows were synchronized in estrus by injecting 25 mg Dinoprost (prostaglandin F2-alpha or PGF2α, 5 ml intramuscular injection of Dinolytic, Pfizer Animal Health Ma Eeig, Broadstairs, Great Britain), a single administration after ultrasonography detecting of the corpus luteum (CL). All cows showed estrus in 2-3 days after PGF2α administration. Also, in days 2 and 9 after induced estrus, a single dose of 2000 IU equine chorionic gonadotropin (PMSG, Folligon, MSD Animal Health, the Netherlands) was administered intramuscularly (i.m.) to each cow. In day 11 of sexual cycle a new dose of PGF2α was administered (Figure 1). The presence of a CL at the beginning of the OPU 2 protocol was confirmed by ultrasonography.



E - estrus
PGF2α - prostaglandin F2-alpha, i.m. injections
PMSG - equine chorionic gonadotrophin, i.m. injections

Figure 1. Protocols for repeated OPU used for Romanian Grey cows (n=10)

2.3. Characterization of follicular deviation

The ovaries were examined with a portable ultrasound device equipped with a trans-rectal linear-array 5-7.5 MHz transducer (WED 3000 V, Well.D Medical Electronics Co. Ltd., Shenzhen, China). Evaluations were performed twice a day (every 12 h) starting at estrus detection until next ovulation. The ultrasonography was repeated in order to determine the time of ovulation, the time of follicle deviation and follicle diameters at deviation and at the time of OPU. During each examination, the size and location of all visible follicles were recorded on a sketch (image stored) of each ovary. Ovaries were mapped by recording the diameters of three largest follicles at each examination. Observed deviation was determined retrospectively by tracking the two largest follicles and classifying them as dominant follicle (DF) and subordinate follicle (SF) (9). The beginning of follicle deviation was defined at the end of a common growth phase, when differences in diameters between the two largest follicles were detected (10). Calculated deviation was performed based on the same data which were used for observed deviation. The segmented regression model consists of two segments (1 and 2) and a Join Point was used for calculated deviation. Segment 1 represented the common growth phase, Segment 2 represented the period of dominance thereafter, and the Join Point represented the beginning of deviation (11).

2.4. Follicular aspiration and oocytes recovery

The animals were restrained in a suitably designed stanchion, which allowed minimal movement. They were prepared for OPU by being administered 100 µg/kg BW Xylazine HCL (Narcoxyl 2, MSD Animal Health, the Netherlands) i.m., followed 10 min later by an epidural anesthesia of 7 ml of 2% Procaine hydrochloride (Procaine 2%, Romvac, Romania).

Transvaginal ultrasound guided oocyte collection was performed using an ultrasound scanner (SSDProSound2, Minitube GmbH, Tiefenbach, Germany) with a convex-sector probe 5 MHz (Bold Medical, Timisoara, Romania), attached to an aspiration pump (Rocket medical, Watfort, England) and ovum pick-up needle (COVA Needle "type A", 17Gx500mm, Minitube GmbH, Tiefenbach, Germany) guidance system. For oocytes aspiration a vacuum of 100 mmHg was used. All follicles >3mm were aspirated and special attention was given to avoid partial aspiration or completely missing of a follicle. Numbers of follicles with different diameters were recorded. Follicles were recorded to be grouped according to size.

The aspirated follicular fluid was collected in 50-ml tubes containing modified OPU recovery medium (Minitube GmbH, Tiefenbach, Germany). Recovered cumulus cell-oocyte complexes (COCs) were morphologically classified into five categories according to criteria previously described by Chaubal & al. (12), as follows: Grade A, >4 layers of cumulus cells; Grade B, three or four layers of cumulus cells; Grade C, one or two layers of cumulus cells; Grade D, denuded oocytes; and Grade E, oocytes with expanded cumulus.

2.5. Statistical analysis

Data was tested for normal distribution using the Shapiro-Wilk test (13). The comparison of data from a single animal over the time period and then a comparison of data between OPU sessions were analyzed by ANOVA. For multiple comparisons, Tukey tests were used. When data had a normal distribution, the homogeneity of variances was determined using the Levene's test (13). The differences were considered to be significant if $P \leq 0.05$. The obtained data are presented in the text as mean ± standard error of the mean.

3. Results

3.1. Follicular deviation

The size of the follicle at which ovulation occurred in the 10 Romanian Grey cows was 12.2 ± 0.1 mm (mean ± S.E.M.) at 72 ± 2.1 h after PGF2α administration. The diameters interval of DF at ovulation was between 11-13 mm.

The future DF (5.1 ± 0.2 mm) was largest compared with the largest SF (4.8 ± 0.2 mm; $P = 0.03$ on the day of ovulation (Day 0). The time of follicle deviation was at 2 days after ovulation when DF (8.1 ± 0.2 mm) was largest ($P < 0.05$) compared with SF (7 ± 0.3 mm). An intense rate of growth for both follicles (DF and SF) in the interval ovulation–deviation could be observed. The growth rate (mm/12 h) of the DF was similar before and after follicle deviation (0.8 ± 0.04 and 0.9 ± 0.08; $P = 0.5$). For SF the growth rate (mm/12 h) was greater before than after deviation (0.6 ± 0.01 and 0.05 ± 0.02; $P = 0.003$). Data concerning time of deviation, and diameter of DF and SF at deviation by observed and calculated methods, is shown in Table 1.

Table 1. Mean (±S.E.M.) characteristics associated with follicle deviation in Romanian Grey (n = 10), using observed and calculated methods to determine deviation.

Characteristics	Observed method	Calculated method	Probability
Time of deviation (Days after ovulation)	2 ± 0.3 (1.6-2)	1.9 ± 0.2 (1.8 – 2)	0.41
DF at deviation (mm)	8.1 ± 0.2 (8-8.7)	7.9 ± 0.4 (7.8 – 8.4)	0.4
SF at deviation (mm)	7 ± 0.3 (6.2 – 7.3)	7.2 ± 0.2 (6.4 – 7.5)	0.58

Day of ovulation = Day 0. Numbers within parentheses represent ranges.
No significant differences were found between observed and calculated methods.

Follicular aspiration and oocytes recovery

A total of 121 and 262 follicles were aspirated during OPU 1 and OPU 2 respectively. Significantly more follicles/cow/session were aspirated in OPU 2 than in OPU 1 ($P < 0.05$) and this applied to all size categories (small: 3 to 4 mm diameter; medium: 5 to 6 mm diameter; large: >7 mm diameter). The higher number of aspirated follicles resulted in a superior number of recovered oocytes for OPU 2 compared to OPU 1 ($P < 0.05$), but also a higher oocyte recovery rate, defined as percentage of COCs recovered per total number of aspirated follicles, could be observed for OPU 2 (Table 2).

The morphological classification of recovered oocytes revealed that OPU 1 protocol yielded higher percentages of grade B and grade C oocytes, whereas OPU 2 offered more oocytes of grades A and D ($P < 0.05$). Only 2% recovered oocytes were classified as grade E and they were collected within OPU 2 protocol.

Table 2. Outcomes of the two OPU protocols performed in Romanian Grey cows

Protocol code*	**OPU 1**	**OPU 2**
Number of animals (n)	10	10
Number of aspiration sessions/cow	2	2
Mean number of aspirated follicles/cow/session	6.2 ± 1.5^{b}	13.1 ± 2^{a}
Categories of follicles at aspiration/cow/session		
3-4 mm	3.2 ± 0.1^{b}	6.1 ± 0.4^{a}
5-6 mm	1 ± 0.5^{b}	4 ± 0.6^{a}
> 7 mm	2 ± 0.3^{b}	3 ± 0.7^{a}
Mean number of oocytes/cow/session	4.20 ± 0.2^{b}	11.36 ± 0.3^{a}
Mean recovery rate** (%)	66.6 ± 1.9^{b}	84.61 ± 2.2^{a}
Oocytes categories		
A	31.3% [b] (25/80)	34.1% [a] (76/223)
B	33.8% [a] (27/80)	30.0% [b] (67/223)
C	32.5% [a] (26/80)	24.2% [b] (54/223)
D	2.5% [b] (2/80)	9.4% [a] (21/223)
E	0% [b] (0/80)	2.2% [a] (5/223)

[a,b] Values followed by different superscripts within rows differ ($p<0.05$).

* OPU 1: estrus detection and OPU sessions on days 6 and 13-14 after its detection; OPU 2: estrus induction with PGF2α and a single administration of PMSG on days 2 and 9 of the cycle, OPU sessions on days 6 and 13-14 after estrus detection, PGF2α on days 11 of cycle.

** Mean recovery rate: number of oocytes/cow/session divided by number of aspirated follicles/cow/session.

4. Discussion

To the best of our knowledge, no studies on stimulation of ovarian follicular growth and follicular aspiration by OPU have been performed so far in Romanian Grey cows. In this study, the sequential monitoring of follicular dynamics after estrus induction with PGF2α and stimulation of ovarian follicular growth with equine chorionic gonadotrophin enabled the characterization of follicular deviation. Also, through repeated OPU sessions it was possible

to establish a favorable protocol for oocytes recovery in a conservation program of genetic resources applied to Romanian Grey cattle.

Selection of dominant follicles in cattle is associated with a deviation in growth rate between the dominant and largest subordinate follicle of a wave (diameter deviation) (14). Our ultrasound monitoring observations suggest that in Romanian Grey cows DF is formed at approximately 2 days after the initiation of a new wave and that the DF reaches its maximum size in approximately 3-4 days. The diameter of the dominant follicle (8.1 ± 0.2 mm) and the largest subordinate follicle (7 ± 0.3 mm) at the time of deviation (2 days after ovulation) in Romanian Grey cows (*B. taurus primigenius*) seemed to be similar with the values reported for Holstein (*B. taurus taurus*) but bigger that in Nelore (*B. taurus indicus*) (15). The time of deviation after wave emergence, and the diameter of DF at the time of deviation did not differ significantly between observed and calculated methods which is in agreement with previous results in *B. t. taurus* (11) and *B. t. indicus* (16). Also, the estrus occurred 2-3 days after PGF2α administration, which is a normal interval for cows (17).

A negative effect of the DF on the competence of oocytes from the subordinate follicles has been described by Hagemann & al. (18). This impairment occurs rather at the non-growing phase of the DF. However, in our study low quality oocytes (grades D and E) were obtained in small proportion during both OPU protocols. This suggests that although the DF is formed on Day 2, its negative effect on the competence of the oocytes of the subordinate follicles does not become apparent before Days 6 or 7, when the subordinate follicles reach more advanced stages of atresia. Apparently, incipient follicular atresia is not detrimental for oocyte competence but, in contrast, it can even be beneficial for it. A plausible explanation for these observations is provided by the so-called prematuration hypothesis, which states that oocytes acquire their developmental competence during a prematuration period (19, 20, 21). This prematuration normally occurs during the last phase of preovulatory development prior to the LH surge but prematuration-like changes also occur during follicular atresia (19, 20, 21). This hypothesis has been supported by observations on oocytes with different cumulus characteristics.

Hormonal stimulation prior to follicular puncture is a common practice associated with OPU in cattle (22). The goal is to obtain a superovulatory response and the protocols usually imply administration of a gonadotropin treatment which causes alterations in follicular development, oocyte maturation and ovulation mechanisms. Although these alterations are generally common to all treatments of superovulation, the final response is also influenced by factors such as the source of commercial preparations of gonadotropin, their purity and the management protocol (23). Of the different hormones that were used over time, such as GnRH (24), FSH (25), eCG/PMSG (26, 27), FSH has usually given the best results in terms of number of follicles aspirated and oocytes retrieved. It has been used successfully in IVP from super-stimulation of follicular growth with OPU techniques in Angus crossbreed cows (12, 28), Holstein cows (29), Simmental heifers (25) and Nelore cattle (30). Although the results are better with FSH, eCG/PMSG is nevertheless often preferred in practice, due to significantly lower price.

Follicular stimulation has proved to be helpful in our study, as OPU 2 protocol generated significantly more follicles to be aspirated than OPU 1. Not only the overall number of follicles was greater in OPU 2, but also the number of follicles assigned to each of the three categories according to their size: large, medium and small. Also more oocytes could be recovered after OPU 2 in comparison with OPU 1. This reflects the beneficial effects of

ovarian stimulation before performing OPU. Nevertheless, it is worth mentioning that a decent number of oocytes could be recovered even without hormonal treatment (OPU 1). We recovered an average of 4.2 oocytes in OPU 1, which is comparable with the average number of eight oocytes per donor usually obtained by OPU in Holstein-Friesian, for example (31). Although it is hardly recommended to perform a rather sophisticated procedure such as OPU without ovarian stimulation that would increase the success chances, it might be applicable in special situations. For example, if the animals are reared in a free system and their restrain for treatment is difficult or simply to avoid the hormonal products, whose excessive use is contraindicated by some authors (32). Thus, the fact that OPU can be applied in Romanian Grey cattle even without hormonal stimulation could represent useful information.

Our study mentions useful aspects of applying reproductive biotechnologies in a rustic but endangered breed of cattle. Preventing ancient breeds from extinction is required for maintaining biodiversity, although their productivity is below modern standards. Their natural resistance against various factors could be used in future genetic selection programs. It is important to take measures that promote a sustainable management of the genetic resources of traditional breeds. This approach involves *in situ* preservation of endangered breeds, use of selection programs to restore the genetic diversity of industrial breeds and protection of the wild relatives that might provide useful genetic resources (33).

The necessity of preserving rustic cattle is being widely acknowledged and concrete steps are currently taken on this matter. However, traditional methods of cattle breeding might not be sufficient for revitalizing populations of ancient cattle breeds, whose decline was dramatic during the last century. The assisted reproductive technologies could be particularly helpful in achieving this goal. They can clearly increase the efficiency of bovine reproduction and their use has grown lately. Further studies should and will continue the work presented in this paper with *in vitro* fertilization of the oocytes obtained through OPU in order to obtain transferable embryos of Romanian Grey.

5. Conclusions

In conclusion, the follicle deviation in Romanian Grey (*B. t. primigenius*) occurred two days after ovulation, and the diameters of DF and SF at deviation were similar with values reported in literature for Holstein (*B. t. taurus*) but bigger that in Nelore (*B. t. indicus*). The administration of equine chorionic gonadotrophin on days 2 and 9 of the sexual cycle in association with a single dose of PGF2α in day 11 represented a good protocol for the stimulation of ovarian follicular growth. The response to OPU procedures offered promising results for the integration of reproductive biotechnologies into animal breeding programs using this robust but endangered breed. Superior results are obtained when ovarian stimulation is performed, but it is noteworthy that OPU can be successfully performed without stimulation too. Future studies should explore the association of OPU with *in vitro* embryo production in order to preserve and increase the populations of Romanian Grey.

Acknowledgements

This study was financed by the research grant PN-II-PCCA-2011-3, Bucharest, Romania "Translating stem cell technologies to the conservation of highly endangered species". The author would like to thank Simona Vlad-Sabie for constructive criticism of the manuscript and for English advice and correction.

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