Intrafollicular oocyte transfer (IFOT): Potential feasibility in the ovine species

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Abstract

Intra-follicular oocyte transfer (IFOT) is a promising and innovative technique for *in vivo* embryo production previously described for equines and bovines. The aim of this study was to assess the feasibility of IFOT in the ovine species. Two preliminary *in vivo* and *in vitro* trials were performed to test the optimal procedures and timing for IFOT. In the *in vivo* trial, follicular growth was monitored with transrectal ultrasonography in ten adult ewes to preliminarily determine the ovulation and ideal timing for IFOT. The *in vitro* trial assessed i) the optimal inner diameter of the injection needle and ii) the recovery rate and integrity of injected cumulus-oocyte complexes (COCs) after follicle aspiration. For IFOT and embryo collection, five ewes were synchronized by CIDR insertion. Forty hours after CIDR removal, in ewes under sedation and general anesthesia, the ovaries were exposed by laparotomy, and the preovulatory follicle was injected with COCs previously collected from ovaries obtained from an abattoir. At 4 h after surgery, fully recovered ewes were housed in a paddock with a ram of proven fertility. Crayon marking on ram's chest was used to detect mating. Ovulation was assessed 40 h after the transfer of oocytes by transrectal ultrasonography. On day 6 after IFOT, embryo collection was performed by uterine flushing. In the *in vitro* testing, injection of >5 mm follicles with a 28 G needle loaded with 30 COCs in a 5 µL volume resulted in higher recovery rates and better preservation of COCs integrity. In the *in vivo* trial, ultrasound scanning revealed that ovulation occurred between 60 and 72 h after CIDR removal in all animals. In one ewe subjected to IFOT, 22/24 oocytes were effectively injected into the preovulatory follicle, but no embryos were collected after flushing. In the remaining four animals, 85/102 oocytes were injected, and six cleaved embryos, 12 morulae and 1 blastocyst were collected, including native embryos. This preliminary

investigation indicated that IFOT in ovine species resulted in ovulation, fimbrial capture, tubal transport of heterologous oocytes and *in vivo* embryo production. Further studies are needed to optimize the embryo recovery rate and develop less invasive techniques for oocyte injection and uterine flushing, such as through a laparoscopic or transcervical approach.

Key words: IFOT, ewe, embryo production, cumulus-oocyte complexes, dominant follicle, follicular injection

1. Introduction

The use of assisted reproductive techniques in modern breeding systems enhances the production of superior genotypes while reducing the generation interval. In ovine species, the commercial application of these technologies is continually increasing but remains performed less frequently than that in bovines. In 2019, according to the data retrieval report of the International Embryo Transfer Society, the worldwide production of *in vivo* and *in vitro* derived bovine embryos was approximately 1.5 million, whereas in sheep, the production of embryos was less than 24,000. Multiple ovulation and embryo transfer (MOET) programs are usually restricted to a few regions (North and South America, and Oceania). Although promising, their efficiency remains debated, because many factors may jeopardize the outcomes in terms of superovulation rate and number of recovered embryos. Among other factors, the choice of hormonal treatment, reproductive status, number of repeated superovulations, breed and lactation have been suggested to affect MOET outcomes [1-4]. Females that do not produce embryos by MOET can still confer high genetic value through laparoscopic ovum pick-up and *in vitro* embryo production (LOPU-IVEP). This technique also has the advantage of allowing efficient progeny production from prepubertal animals [5], thus accelerating genetic gain. In contrast to MOET, it can potentially be repeated several times on the same donor [6] (Morton et al. 2005), thus yielding in a higher number of embryos per animal. However, its use as a tool for genetic improvement is limited because it requires expensive instruments, trained veterinarians and laboratories equipped for *in vitro* embryo production [7].

In addition to the above described techniques, intrafollicular transfer of oocytes (IFOT) may be a promising and innovative technique for *in vivo* embryo production. It consists of preovulatory follicle injection of heterologous oocytes. which undergo ovulation and fertilization, and eventually develop to preimplantation embryonic stages. Embryos are finally recovered by uterine flushing [8]. This procedure was first described by Fleming et al. (1985) in baboons and cattle. In bovines, additional embryos have been collected after the intrafollicular injection of oocytes derived by ovum pick-up [9]. After transfer, these embryos have successfully resulted in the birth of healthy calves [10]. In mares, this technique was first described by Hinrichs and Di Giorgi (1991) [11], but it is not considered repeatable or clinically applicable [12]. To our knowledge, IFOT has never been performed in small ruminants. Therefore, the aim of this study was to conduct a preliminary investigation of the feasibility of this technique in ovine species by using a laparotomic approach, to assess whether injection of immature cumulus-oocyte complexes (COCs) into a pre ovulatory follicle would result in ovulation, fimbrial capture, fertilization and eventually embryo development.

2. Materials and methods

2.1 Experimental design

The experiment consisted of two preliminary tests designed to assess the optimal procedures and timing for IFOT and an *in vivo* experiment in which oocyte transfer was performed on live animals (Fig. 1). The study was conducted on the premises of the farm of the Department of Veterinary Medicine in Sardinia, Italy, located at 40° 42′ N; 8° 33′ E. All procedures followed the European regulations for the Care and Welfare of Animals in Research and were approved by the organization in charge of animal welfare and animal testing (Organismo Preposto al Benessere Animale ed alla Sperimentazione sugli Animali-OPBSA) of the University of Sassari and the Italian Ministry of Health (authorization no. 614/2020-PR). For this purpose, 15 Sarda sheep (age 2–3 y; body weight 30–35 kg) were randomly allocated to one of two experimental groups. Group one included ten animals in the preliminary test (to determine the time of ovulation and the ideal time for oocyte transfer), and group two included five ewes for IFOT and embryo collection.

2.2 Preliminary tests

2.2.1 *In vivo*

In group one, estrus was synchronized by insertion of an intravaginal device impregnated with progesterone (CIDR Ovis® 350 mg, Zoetis, Italy) for 12 d. At the time of CIDR removal, the animals received 333 IU of PMSG (Folligon®, MSD Animal Health S.r. L., Italy) IM. The timing of follicular growth and ovulation was monitored by transrectal ultrasonography (MyLab One, Esaote, Italy, frequency 10 Mhz, depth 5 cm) with a linear probe (7.5 Mhz, SV3513 VET, Esaote, Italy) at 0, 24, 48, 60 and 72 h after CIDR withdrawal.

2.2.2 In vitro

Ovaries were collected from slaughtered adult Sarda ewes and within 1–2 h were transported to the laboratory in Dulbecco's phosphate buffered saline (PBS) with antibiotics at 30°C. Collection of COCs was performed by gently slicing with a surgical blade half of the collected ovaries in sterile Petri dishes containing 20 mM HEPES-buffered TCM 199 supplemented with 0.1% (wt/v) polyvinyl alcohol and antibiotics at 38°C. Only COCs showing several intact cumulus cell layers and uniform cytoplasm were selected for the experiment. The remaining ovaries were carefully inspected for follicles >5 mm in diameter and used for the injection of 30 COCs previously collected and grouped in 5 µL TCM 199. The transfer into the follicles was performed by aspiration of the medium containing COCs with insulin syringes (1 mL) fitted with needles of different gauges (27 G, 28 G and

30 G) with a bevel angle of 15°. The follicle was then re-punctured, and its contents were aspirated with a 23 G needle to assess i) the optimal inner diameter of the injection needle and ii) the recovery rate and integrity of injected COCs after follicle aspiration. These procedures were carried out in a total of 45 follicles.

2.3 Intrafollicular oocyte transfer (IFOT)

In ewes of group two (n=5), estrus synchronization was performed as in group one, and IFOT was performed at 40 h after CIDR removal [8].

Before surgery, the ewes were not given access to food and water for 24 h and 12 h, respectively. All animals were subjected to sedation (fentanyl 2.5 µg/kg body weight + diazepam 0.3 mg/kg body weight), induction (lidocaine 2 mg/kg body weight + pentothal sodium 3 mg/kg body weight), and general anesthesia was maintained with 8.6% desflurane (Dräger Fabius® GS; Fig. 2). The ewes were then placed in a cradle in dorsal recumbency, and the abdominal area was surgically scrubbed with an antiseptic solution. The preovulatory follicle was visualized after the uterus and ovaries were exteriorized through a midventral incision (Fig. 3A), and its diameter was recorded with a sterile surgical ruler. The selected follicle was then punctured with an insulin syringe with a 28 G fixed needle that had been loaded with 5 µL PBS supplemented with 10% fetal calf serum (FCS) at 38°C, containing 22–28 COCs. Cumulus oocytes complexes were obtained from ovaries collected from a local slaughterhouse as described above. Ovarian slicing and COC selection started approximately 30 min before sedation. Only COCs with at least three layers of cumulus cells and uniform cytoplasm were selected and retained for injection. The content of the syringe was carefully injected into the follicle (Fig. 3B). After retrieval, the needle was immediately flushed with 1 mL PBS to assess the number of oocytes effectively injected into the follicle. The ovaries and uterus were irrigated copiously with sterile saline solution before being returned to the abdominal cavity. Postsurgical care consisted of antibiotics (benzylpenicillin at 40.000 IU/kg and dihydrostreptomycin at 50 mg/kg, body weight/day) and anti-inflammatory drugs (ketoprofen at 3 mg/kg, body weight/day) administered for four consecutive days. At 4 h after surgery, full recovery was verified, and the animals were transferred to a paddock with a ram of proven fertility wearing a marking harness. Crayon marks were assumed to indicate mating. In marked ewes, transrectal ultrasonography was performed 40 h after the transfer to evaluate whether ovulation of the injected follicle occurred, then 5 d after the transfer to assess the presence of a functional corpus luteum.

2.4 Embryo collection and evaluation

Embryos were surgically recovered on day 6 after mating. Sedation and anesthesia procedures were the same as those used for IFOT. After laparotomy and exposure of the uterine horns, a two-way silicone Foley catheter was inserted at the base of the uterine horn, ipsilateral to the corpus luteum, and cuffed (Fig. 4). A volume of 20–40 mL of PBS supplemented with 5% FCS was introduced into the tip of the uterine horn with a 19 G intravenous cannula. The flushing medium was collected in an embryo filter through manual massage of the uterus. The same procedure was repeated on the contralateral horn. The uterus was washed with saline and returned to the abdominal cavity. The flushing medium containing presumptive embryos was transferred to a Petri dish to be examined under a stereomicroscope (Olympus SZX7, Olympus Optical Co., Italy) for classification of recovered structures.

3. Results

3.1 Preliminary testing

Ultrasound monitoring of follicular growth and the time of ovulation revealed that four ewes ovulated within 60 h, and the remaining six ovulated within 72 h after CIDR removal. From 24 h onward, in 9/10 ewes had at least one follicle with a diameter of >5 mm that underwent ovulation, reaching a diameter of 7.5–8 mm (Fig. 5).

3.1.2 *In vitro*

The results of the *in vitro* testing are reported in Table 1. Six follicles were injected with a 27 G needle and a syringe loaded with 5 µL of medium containing 30 COCs. Reflux of fluid from the punctured follicles was observed, and the recovery rate of injected oocytes after aspiration was 38%. In 14 injections with a 30 G needle, the recovery rate increased to 68%, and no reflux was observed. However, all recovered oocytes were partially or completely denuded. A further 25 injections were performed with a 28 G needle: the recovery rate was 75.5%, and better preservation of the integrity of COCs was observed. Because we estimated that 5 µL was the minimum volume of medium that could contain 30 oocytes, and 28 G was the most suitable needle diameter for COC integrity and reflux prevention, this procedure was chosen for IFOT *in vivo*.

Injections performed (n)	*Needle gauge (G)*	*Volume (µL)*	*Recovery rate*	*Observations*
6	**27**	5	**38%**	Reflux
25	**28**	5	**75.5%**	-
14	**30**	5	**68%**	Stripped

Table 1: Results of preliminary *in vitro* testing to evaluate the most suitable needle gauge for injection in terms of the recovery rate and integrity of COCs.

3.2 Intrafollicular oocyte transfer (IFOT)

The results of IFOT are shown in Table 2. During injection in the preovulatory follicle, the number of oocytes that were not successfully transferred inside the follicle ranged between 5-13/30. All non-injected oocytes were recovered after needle washing. Mounting of the harnessed ram was confirmed in all ewes within 12 h after IFOT. Ovulation of the injected follicle and the presence of one functional corpus luteum were confirmed by transrectal ultrasonography in all ewes, at 40 h and 5 d after the transfer, respectively.

Including the native oocytes, uterine flushing at day yielded 19 embryos (six cleaved, one blastocyst and 12 morulae) over 107 injected oocytes (17.7%) and seven degenerated oocytes (6.5%).

Sheep no.	*IFOT*				*FLUSHING*			
	Diameter of injected follicle (mm)	*Oocytes loaded in the syringe*	*Effectively injected oocytes*	*Oocyte loss rate (%)*	*Degenerated oocytes*	*Cleaved embryos*	*>8 cell embryos*	
							Morulae	*Blastocysts*
1	5	22	20	9	0	1	2	0
2	5	25	17	32	2	0	2	0
3	6	24	22	8,3	0	0	0	0
4	7	27	25	7,4	1	2	7	1
5	6	28	23	17,8	5	3	1	0
Mean		**25.2**	**21.4**	**14.9**	**1.6**	**1.2**	**2.4**	**0.2**
Total		**126**	**107**		**8**	**6**	**12**	**1**

Table 2: Outcomes of the IFOT procedure in the ovine, performed 40 h after CIDR removal and of the uterine flushing at day 6 after mating. Native embryos are included in the count.

4. Discussion

To our knowledge, this is the first study in literature reporting the feasibility of IFOT in the ovine species. Ovine heterologous immature oocytes, when injected *in vivo* into a preovulatory follicle, can undergo maturation, ovulate, be captured by fimbriae and fertilized, and cleave up to more than eight-cell stage embryos. However, the double laparotomic approach (injection of oocytes and embryo recovery) limits the application of the protocol. Intrafollicular oocyte transfer has been described for bovine and equine species, and *in vivo* embryo production has been reported in both species [8,11-13]. According to our experimental results, the choice of the best option for *in vivo* follicular injection in sheep was based on several parameters. The volume of medium (5 mL) was chosen as the minimum amount of TCM99 that could contain a maximum of 30 COCs and avoid perturbation of the intrafollicular environment and the total volume of follicular fluid. The needle diameter (28 G) was selected on the basis of the recovery rates after injection and the integrity of recovered COCs. Larger diameters resulted in leakage of fluid from the punctured follicles and a loss of COCs, whereas smaller diameters (e.g., 30 G) caused structural damage to the injected COCs and a loss of granulosa cells. The integrity of COCs was an important parameter, given that the close coupling between oocyte and cumulus cells is critical for developmental competence [14]. The choice of the number of injected oocytes was based on previous studies in bovine species. Spricigo et al. have injected a maximum of 25 oocytes in the preovulatory follicle [8], whereas Kassens et al. have used larger numbers (i.e., 60 COCs; [10]). Given the small size of the dominant follicle in sheep, we estimated that 20–30 oocytes should be appropriate for avoiding perturbation of ovulation mechanisms and fimbrial capture. This estimate is supported by Simoes et al. (2021), who have hypothesized that injecting large numbers of oocytes in the dominant follicle might compromise oocyte release and uptake by fimbriae [15]. Moreover, given that this was the first attempt of IFOT in ovine, we chose to inject freshly collected COCs from slaughtered ewes to transfer high quality selected oocytes, thus avoiding variability in oocyte competence and embryo production. Several attempts were made to enhance embryo production by IFOT, with different sources of oocytes and different stages of maturation. In bovines, a recent study has compared embryo yield after transfer of *in vitro* matured oocytes (IVM) or those matured *in vivo* after 20 h or 28 h [15]. The authors reported no significant differences among groups in terms of embryo production, but interestingly described higher fertilization rates for oocytes matured *in vivo* for 20 h than for IVM oocytes [15]. Similarly, Spricigo et al. (2016) have suggested that, to provide sufficient time for the maturation of injected oocytes, IFOT should be performed at least 18 h before ovulation [8]. These observations suggest that, although the timing of the estrus cycle and ovulation differs between bovine and ovine species, this

amount of time in a physiological preovulatory follicular microenvironment is suitable to achieve maturation of heterologous oocytes. The beneficial effects of follicular fluid on oocyte maturation have been extensively reported. Supplementation of follicular fluid in *in vitro* culture medium enhances developmental competence in lamb oocytes [16] and promotes cytoplasmic and nuclear maturation of IVM oocytes in several species (bovine [17], ovine [18] and porcine [19]). Follicle diameter at the time of IFOT appears to be another crucial factor affecting outcomes in terms of embryo production. Kassens et al. (2015) have reported significantly higher numbers of extra embryos for IFOT performed in follicles of 13–14 mm than in follicles of 9 to 10 mm [10]. Although the number of animals used in the present experiment was limited and insufficient to draw a conclusion, the highest embryo yield was obtained by IFOT in a follicle of 7 mm. Follicular fluid composition changes in relation to follicular size, such that larger follicles contain more glucose and cholesterol [20]. We speculate that the progressive increase in follicular size close to ovulation may correspond to the optimal fluid composition in terms of hormones and metabolites, which is suitable for nuclear and cytoplasmic maturation, fertilization and embryonic development of heterologous oocytes.

In the *in vivo* preliminary testing, the time between the removal of the progesterone device and ovulation (60 to 72 h) was similar to that reported for this species by Martinez-Ros and Gonzalez-Bulnes (2019) [21], and the size of the dominant follicle at 40 h was approximately 6 mm. On the basis of these observations and considering that heterologous oocytes needed to remain inside the follicle for at least 20 h before ovulation to achieve maturation, we performed IFOT 40 h after the device removal, on follicles of 5–7 mm in size. Another important aspect is that manipulation of the dominant follicle and injection did not interfere with sexual behavior or with the physiological mechanisms of ovulation and subsequent corpus luteum development. In heifers, follicle puncturing for oocyte aspiration may compromise CL functionality, thus decreasing the size and progesterone production [22]. However, fully functional CLs have been described after IFOT [8, 13]. In our study, ultrasound assessment of the size and blood supply revealed no alterations in the CL, although circulating progesterone measurement for better evaluation of functionality was not performed.

The recovery rate (25.3%, including both degenerated oocytes and embryos) was similar to that reported by Spricigo et al. in bovines (25.9%; [8]) but lower than those reported by Kassens et al. (35.2%; [10]) and Hoelker et al. (38.5%; [13]). The lower recovery rate of this preliminary study might have been a result of the above-mentioned species-specific differences and the objective difficulties in performing IFOT in ovines, which required a laparotomic approach for both injection and uterine flushing. Moreover, although the number of animals in the study was low, high variability among individuals was observed. Many factors might have affected the outcomes, and further

investigations are necessary to design a repeatable and successful IFOT protocol in terms of embryo yield. Better standardization in the size of the dominant follicle may help improve the procedure. With the current design, IFOT also has several limitations that must be overcome to allow its use in commercial breeding programs. Alternatives to the invasive surgical procedures of injection of oocytes and uterine flushing are crucial to make IFOT feasible in field conditions for ovine species. First, as in MOET protocols, the superovulatory response in donors of superior genotypes must be standardized to prevent individual variability; second, a laparoscopic approach for COC collection and injection in recipients, and transcervical uterine flushing is preferable. However, currently repeatable protocols for these techniques are lacking [23,24].

In conclusion, a preliminary protocol for IFOT in ovine species was designed, and the maturation, ovulation and fertilization of heterologous oocytes and embryo production are described. Intrafollicular oocyte transfer would provide a valid technique for *in vivo* embryo production in ovine species. Although promising, the procedure had poor repeatability. Further investigations will be mandatory to assess the feasibility of routine IFOT application.

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Figure captions

Fig. 1: Experimental design. Two preliminary *in vitro* and *in vivo* tests were performed to assess the optimal conditions for performing IFOT on live animals. Intrafollicular oocyte transfer was performed on five ewes at 40 h after CIDR removal, and presumptive embryos were flushed 6 d after mating with fertile rams.

Fig. 2: **Preparation of animals for surgery**. After sedation and induction, ewes were intubated, and general anesthesia was maintained with 8.6% desflurane.

Fig. 3: Intrafollicular oocyte transfer. A) After laparotomy**,** uterine horns and ovaries were exteriorized to locate and measure the size of the preovulatory follicle (black arrow). B) The follicle was gently punctured, oocytes were transferred, and the needle was carefully retrieved.

Fig. 4: Uterine flushing for embryo collection. At day 6 after mating, uterine horns were flushed with PBS + 5% FCS, and presumptive embryos were collected in a Petri dish.

Fig. 5: Ultrasound monitoring of the growth of the dominant follicle. After synchronization with CIDR and PMSG, ewes ovulated when the dominant follicle reached >5 mm in diameter, from 48 h after CIDR removal onward. Error bars represent standard deviations.

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Figure 1

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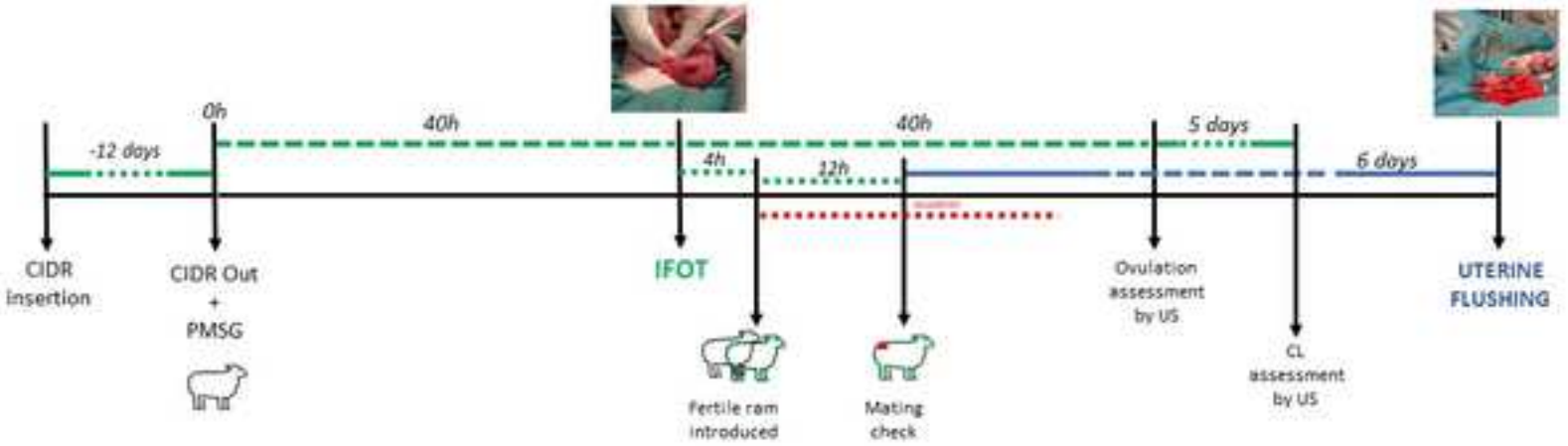

Figure 2

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Figure 3

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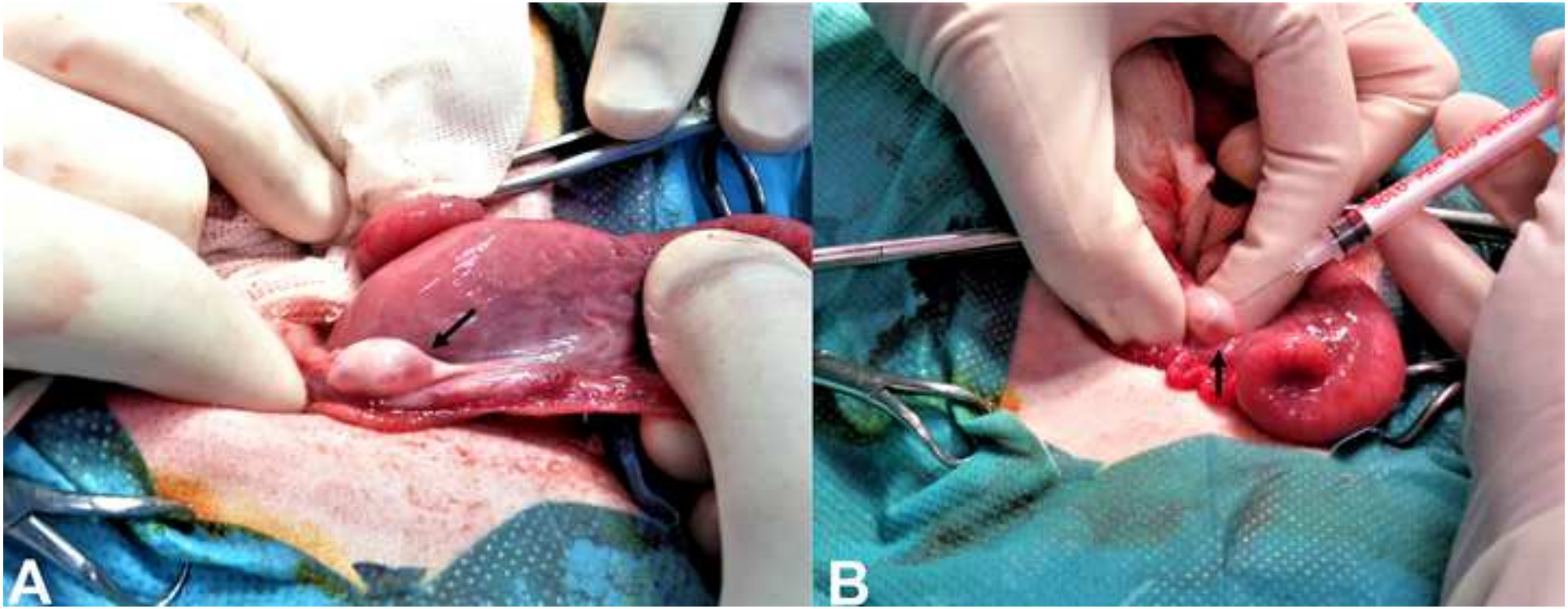

Figure 4

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Figure 5

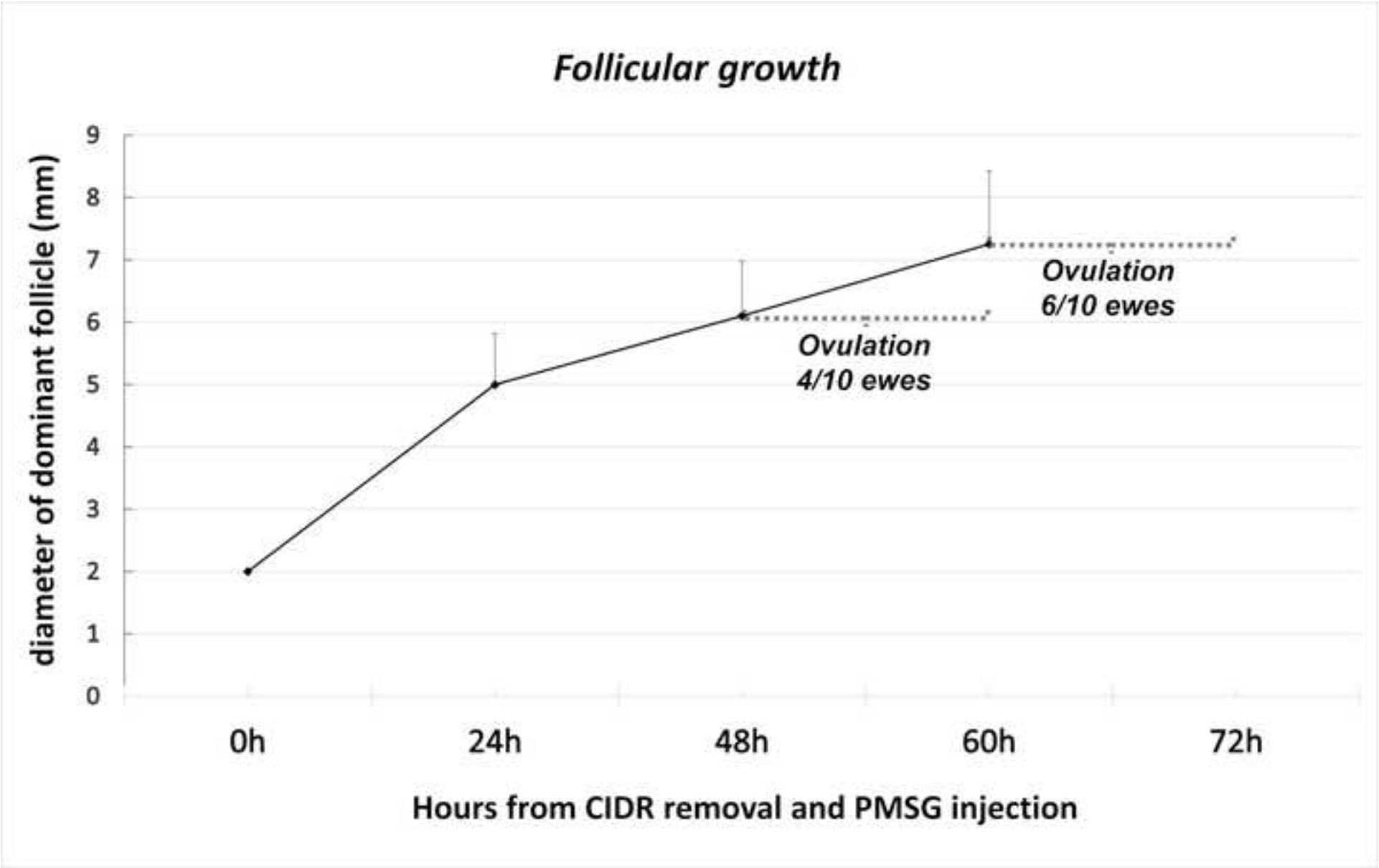

Fig.5.jpg