Original article

The repeatable method of laparoscopic ovum pick-up (OPU) in sheep: clinical aspects and efficiency of the method

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Abstract

The aim of the study was to develop new laparoscopic technique for repeated recovery of sheep oocytes. Oocytes were aspirated with specifically designed catheter. It allowed to recover oocytes without ovary damage and to preserve very good quality of recovered oocytes. Fifteen ewes were oocytes donors. Oocytes were collected: one time (group I, n=15), two times (group II, n=15), three times (group III, n=10), four times (group IV, n=5). The endoscope was inserted into the abdominal cavity. Two trockars for putting the manipulators were inserted 15 cm cranial from the udder. Oocytes were collected by aspiration of the follicular fluid from the ovarian follicles. The observed clinical complications were: ovary bleeding and cicatrix at place of needle insertion, the fragmentary adhesion of infundibulum and ovary, adhesions of omentum and peritoneum near the place where the grasping forceps were inserted and adhesion of ovary and uterus. Ovarian follicles (n=204) were aspirated, 130 (63.8%) oocytes were obtained. Out of 130 obtained oocytes, 112 were qualified for *in vitro* maturation. The remaining 18 oocytes (13.8%) were rejected due to cytoplasmic changes. The proposed technique allows for the collecting oocytes of good quality that can be used for IMV/IVF techniques and cloning.

Key words: ovum pick-up, sheep, laparoscopy, oocytes

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Introduction

During last few years, the rapid progress in biotechnology techniques was made, concerning i.a. fertilization and embryo obtaining *in vitro* (*IVF/IVM - in vitro fertilization / in vitro maturation*), embryos storing in low temperatures, obtaining of monozygotic twins, chimeras, transgenic animals for breeding and medical purpose, as well as embryos sexing and cloning. Development of these biotechnology techniques would not be possible without simple and efficient methods of oocytes recovery, with the possibility of its common practical application. Ethical aspects are also extremally important. The legal regulation of animal welfare and restrictive requirements of animal utilization in scientific research forced scientists to develop minimally invasive techniques, like laparoscopy. Therefore, it was necessary to develop and apply new techniques for the repeatable, noninvasive and efficient recovery of oocytes in living donors, which would be able to replace surgical or post-slaughter methods. The alternative for these invasive procedures are non-invasive methods, as non-surgical, so called transcervical or bloodless methods and low-invasive methods, called endoscopic or laparoscopic. The essence of non-surgical (bloodless) methods is obtaining and transferring oocytes through cervix, without surgical intervention, with employment of original device or adapted methods of artificial insemination. Essentials of low-invasive methods is limitation of surgical intervention to the absolutely necessary level, with employment of endoscopic and laparoscopic methods. The use of laparoscopy and video surgery enabled the laparoscopy ovum pick-up (LOPU) technique to be used in medium-sized farm animals such as sheep, goats and pigs (Stangl et al. 1999, Baldassarre and Karatzas 2004, Teixeira et al. 2011, Souza-Fabjan et al. 2013, Wieczorek et al. 2015). The use of this technique enable boodless, safe and quick access to abdomen organs and simply ovary stabilization and aspiration of ovarian follicles (Kühholzer et al. 1997, Teixeira et al. 2011). In comparison with surgical methods, laparoscopy enables animals to come back to herd immediately after operation and it may be used in farm conditions (Bari et al. 2000). It allows to restrict time necessary for operation (Nellenshulte and Niemann 1992, McMillian and Hall 1994). It is simple and safe to reach ovary or middle and initial part of uterus horn, to make multiple operation in the same animal and to minimize doses and to shorten time of usage anesthetic and analgesic drugs (Teixeira et al. 2011). It helps to eliminate secondary complications of surgery (McMillan and Hall 1994, Vrisman 2014) and to minimize risk of transmission of infectious diseases, to limit costs of animal transport and in principle to make the recovery of genetic material of very good quality easier (Bari et al. 2000). This method is limited by its relatively low and variable efficiency, allowing 50-80% of oocytes to be recovered from aspirated ovarian follicles and 30-90% of them to be accepted for maturation and fertilization *in vitro*. The efficiency depends on the good selection of donors, hormonal stimulation, frequency of oocyte recovery and technical factors (Baldassarre et al. 1994, 1996, Kühholzer et al. 1997, Stangl et al. 1999, Teixeira et al. 2011, Padilha et al. 2014).

The aim of the study was to develop laparoscopic technique for repeated recovery of sheep oocytes, useful for culture, fertilization *in vitro* and cloning. As clinical aspects, bleeding, tissue damage, adhesions in abdominal cavity, scar formation and disorders of reproductive tract in repeated donors after 2 – 4 operations of recovering oocyte were taken into consideration.

Materials and Methods

All procedures were performed with the prior approval of II Animal Ethics Committee in Cracow, approval number 453/2007.

The study was done in 15 sheep of wrzosowka breed. To exclude the influence of extraneous factors on the experiment, the animals from one herd (over 300 animals)were chosen. They were kept in one place from birth, in the same breeding condition. Animals for experiment were chosen randomly. They were fed good quality hay ad libitum as well as complete substantive feed about 0.3 kg per day, with constant access to fresh drinking water. Groups were assigned according to the frequency of oocyte collection: one collection (Group I, n=15), two collections (Group II, n=15), three collections (Group III, n=10) and four collections (Group IV, n=5). The mean time interval between sconsecutive recovery session was 43, 54 and 59 days, for intervals between 1st and 2nd procedures (group I and II), 2nd and 3rd procedures (group II and III) and 3rd and 4th procedures (group III and IV), respectively.

The animals were synchronized and superovulated. For estrus synchronization, the animals were treated with intravaginal sponges of Chronogest CR (Flugestone 20 mg, Intervet, the Netherlands) for a total of 11 – 14 days. To induce superovulation, the donors received intramuscular injection of 1000 U.I. PMSG (Serogonadotropin, Biovet, Pulawy, Poland). PMSG was administered 16 – 24 hours prior to sponge removal. The oocytes were collected 24 hour after sponge removal.

Laparoscopic ovum pick-up was done under general anaesthesia. Animals were premedicated with intra-

Table 1. The evaluation of clinical symptoms and complications after 1–4 laparoscopic ovum pick-up (L-OPU) session in sheep.

Group (n = number of ovaries)	Clinical symptoms				
	Ovary bleeding at place of needle insertion	Ovary cicatrix at place of needle insertion	Fragmentary adhesion of infundibulum and ovary	Adhesion of omentum and peritoneum next to the location of grasping forceps insertion	Adhesion of ovary and uterus
I (n = 30)	10 (33%)[b]	-	-	-	-
II (n = 30)	14 (47%)	17 (57%)[a]	1	-	-
III (n = 20)	12 (60%)[b]	14 (70%)[b]	-	1	1
IV (n = 10)	6 (60%)	8 (80%)[c]	1	1	1
Total (n = 90)	42 (47%)	39 (43%)	2	2	2

[a] – statistically significant difference between group I and II, $p \leq 0.05$;
[b] – statistically significant difference between group I and III, $p \leq 0.05$;
[c] – I and IV, $p \leq 0.05$

muscular injection of atropine 0.05 mg/kg (Atropinum Sulfuricum 0.5 mg, Polfa Warsaw, Poland) and xylazine 0.05–0.1 mg/kg (Xylavet 2%, Alfasan International B.V., Netherlands). The anaesthesia was induced with intravenous injection of thiopentone natrium 5–10 mg/kg (Tiopental 1.0 g, Sandoz GmBH, Austria). The anaesthesia lasted 15–30 minutes.

The anaesthetized animals were immobilized in a cradle in dorsal position. The sheep were then hung with their head down on a mobile surgery table at an angle of approximately 45°. The trocar for endoscopic camera was inserted into abdominal cavity on the left side of abdominal midline, about 15 cm cranial from the udder. Next two trocars for atraumatic grasping forceps were inserted after filling abdomen cavity with filtered air. One trocar was inserted on the right side from the midline and about 15 cm cranial from the udder, the second one in abdominal midline about 30–35 cm cranial from the udder. These three trocars were inserted into abdominal cavity on an isosceles triangle plan, with sides of 15-20 cm. The basis of triangle was 15 cm in cranial direction from the udder. The ovary was isolated from the infundibulum and stabilized by holding mesosalphinx as close to its end as possible.

Oocytes were recovered by aspiration of the follicular fluid from the ovarian follicles (Fig. 1). Oocytes were aspirated with originally designed catheter 25 cm long, 5 mm diameter, with 10 mm long needle, 21–22G thin, bevel 30°. It allows to recover oocytes without ovary damage and to keep very good quality of recovered oocytes. The aspirated fluid was stored in a Petri dish containing Medium 199 HEPES Modification (Sigma-Aldrich, Cat. No. M2520) supplemented with 0.5 IU/ml heparin (Heparin 5000 I.U./ml, Biochemie Austria). The catheter, grasp forceps and the endoscope were removed in order reversed from the one they were installed. The peritoneum and the abdomen wall were not sutured. The single harmonic suture was put on skin. The duration of operation was 18 – 25 min, on average it was 20 min. The animals were observed for 14 days after operation. All animals were in good general condition.

The recovered oocytes were flushed three times with a Medium 199 HEPES Modification (Sigma-Aldrich, No kat. M2520) supplemented with 0.5 IU/ml heparin (Heparin 5000 I.U./ml, Biochemie Austria) and examined under a stereoscopic microscope (25-40x). The following rules of the evaluation and classification of the oocytes were established: class I – homogenous cytoplasm, at least 3 layers of granulosa cells, class II – homogenous cytoplasm, 1-2 layers of granulosa cells, class III – homogenous cytoplasm, no granulosa cells, class IV – heterogenous cytoplasm, independing of the granulosa cells. Oocytes class I, II and III were qualified for the culture.

Statistical analyses were conducted using the Statistica 8 software (StatSoft Inc.). The differences between

Table 2. Number of aspirated ovaries, ovarian follicles, recovered oocytes and number of recovered oocytes qualified and not qualified for maturation in sheep after one, two, three and four times of OPU. The morphology of recovered oocytes, distribution of the 4 classes oocytes in sheep after one, two, three and four times of OPU. Oocytes classes I – III qualified for maturation, class IV not qualified for maturation.

Group (n = number of animals)	Number of aspirated ovaries	Number of aspirated ovarian follicles	Number of recovered oocytes (%)	Number of recovered oocytes/ /one sheep	The morphology of recovered oocytes				
					Class I Number of oocyte (%)	Class II Number of oocyte (%)	Class III Number of oocyte (%)	Number of oocytes qualified for maturation Class I + II+ III (%)	Class IV Number of oocyte - not qualified for maturation (%)
I (n = 15)	30	59	37 (62.7 ± 0.39)	2.5 ± 1.68	16 (43.2 ± 0.45)[a,b]	15 (40.5 ± 0.44)	0 (0.0)[a]	31 (83.8 ± 0.41)	6 (16.2 ± 0.30)[b]
II (n = 15)	26	70	45 (64.3 ± 0.24)	1.7 ± 0.83	20 (44.4 ± 0.38)[c,d]	14 (31.1 ± 0.42)	6 (46.7 ± 0.18)	40 (88.9 ± 0.29)	5 (11.1 ± 0.29)
III (n = 10)	18	49	32 (65.3 ± 0.33)	3.2 ± 2.7	4 (12.5 ± 0.31)[a,c]	22 (68.8 ± 0.45)	3 (9.4 ± 0.12)	29 (90.6 ± 0.47)	3 (9.4 ± 0.12)
IV (n = 5)	10	26	16 (61.5 ± 0.1)	3.2 ± 1.3	0 (0.0)[b,d]	5 (31.3 ± 0.45)	7 (43.8 ± 0.45)	12 (75.0 ± 0.42)	4 (25.0 ± 0.42)
Total (n = 45)	84	204	130 (63.7 ± 0.21)	2.9 ± 1.7	40 (30.8 ± 0.38)	56 (43.1 ± 0.41)	16 (12.3 ± 0.15)	112 (86.2 ± 0.38)	18 (13.8 ± 0.31)

[a] – statistically significant difference between group I and III, $p \leq 0.,05$;
[b] – statistically significant difference between group I and IV, $p \leq 0.05$;
[c] – statistically significant difference between group II and III, $p \leq 0.05$;
[d] – statistically significant difference between group II and IV, $p \leq 0.05$,

Table 3. The correlation between number of sessions, time between sessions and quantity and quality of recovered oocytes.

	Number of recovered oocytes	Oocytes class I	Oocytes class II	Oocytes class III	Oocytes class IV	Oocytes qualified for IVM
Session number	0.06	-0.34	0.01	0.48	0.15	-0.05
Time between session	0.16	-0.43	0.006	0.17	0.33	-0.31

$p \leq 0.05$, n = 45

groups were analyzed by Student's t-test and the correlation coefficient was determined by the Paerson's test. T-studet test was used to analyze the difference in number and quality of obtained oocytes between groups. It was the way to evaluate the influence of subsequent procedure to the number and quality of obtained oocytes. This test was also used to analyse the difference in occurring the defined lesions and complications after the procedure, what let us define the influence of subsequent procedures on complications afterwards. Paerson's test was used to define the correlation between the number and quality of obtained oocytes and the subsequent procedures as well as the time between them.

Results

The results are presented in Tables 1-3 and Figs. 1, 2.

The evaluation of clinical symptoms after laparoscopic OPU session is presented in table 1. Small to middle bleeding from aspirated ovarian follicle was observed at place of needle insertion in all groups, on average in 50% of ovaries (33–67%) (Fig. 2A,B). Statistically significant difference was observed only between groups I and III. Group I showed no other clinical symptoms. In group II, III and IV small cicatrices on ovaries were observed. On average, cicatrices were observed in 72% of ovaries (65–80%). There was statistically significant difference between groups I–II, I–III and I–IV. No difference between group II–III and III–IV was observed. One sheep after second recovery from group II developed the unilateral fragmen-
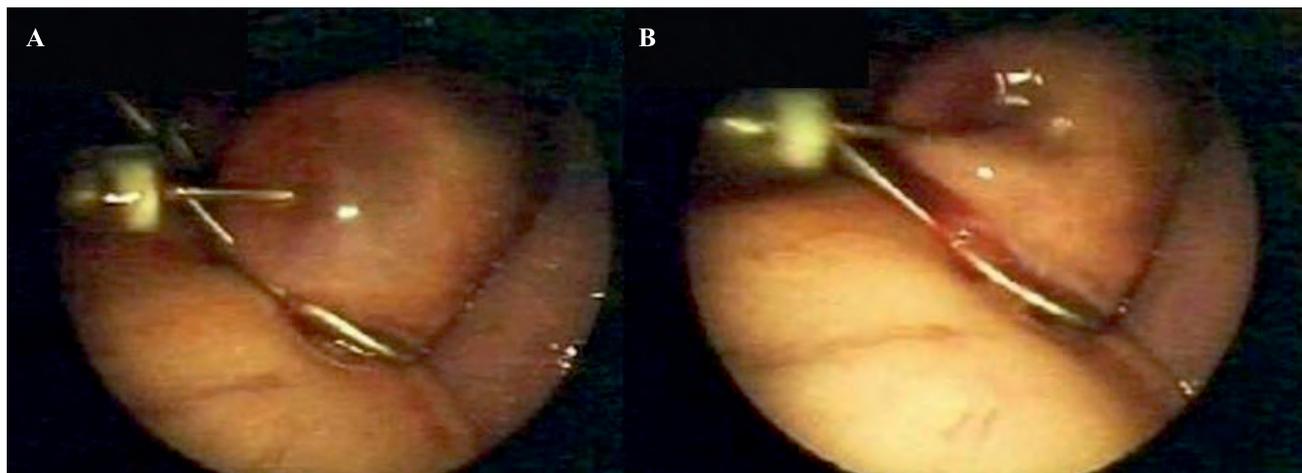
Fig. 1. Aspiration of the follicular fluid (A. – ovary follicle before aspiration, B. – ovary follicle immediately after aspiration).
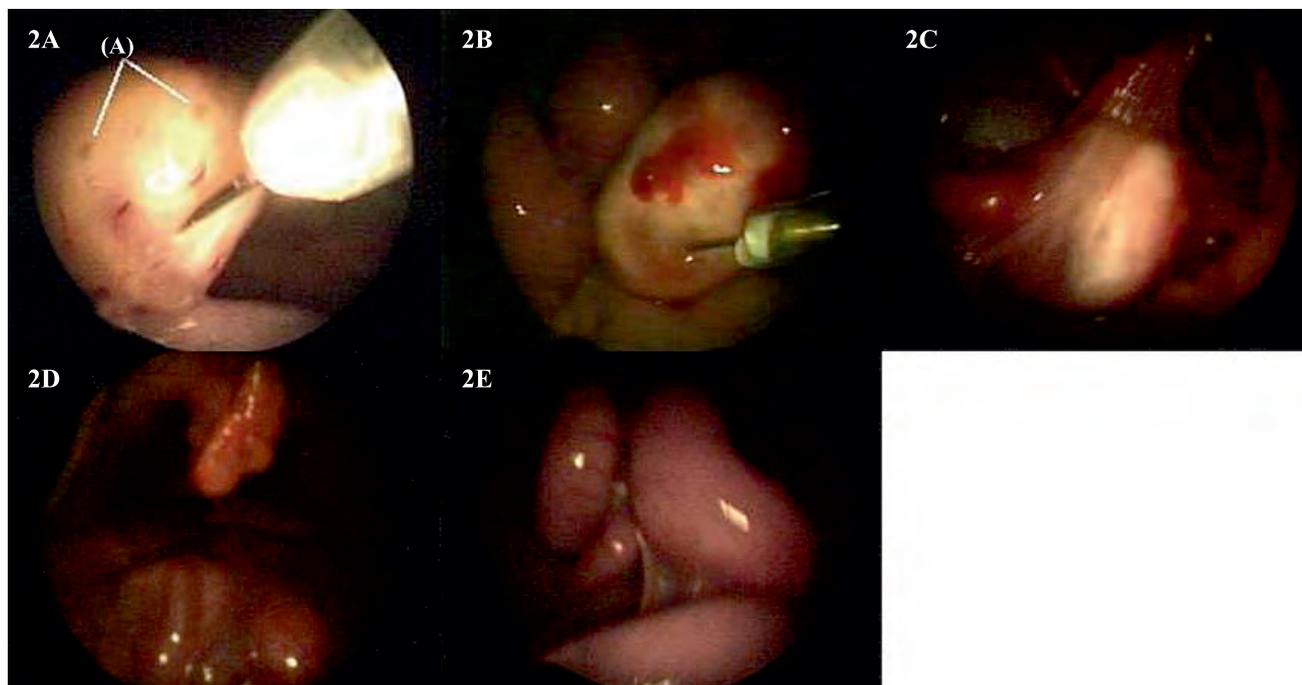

Fig. 2. The complications after laparoscopic OPU in sheep. Fig. 2A - Places of needle insertion after aspiration and ovary cicatrices after previous aspirations (A), Fig. 2B - ovary bleeding at place of needle insertion during aspiration of ovarian follicle, Fig. 2C - fragmentary adhesion of infundibulum and ovary, Fig. 2D - adhesions of omentum and peritoneum next to the location of grasping forceps insertion, Fig. 2E - fragmentary adhesion of ovary and uterus.

tary adhesion of infundibulum and ovary (Fig. 2C). One sheep from group III developed adhesion of omentum and peritoneum next to the place where grasping forceps were inserted (Fig. 2D) and the other sheep developed adhesion of ovary and uterus (Fig. 2E). The fragmentary adhesion of infundibulum and ovary, adhesion of omentum and peritoneum next to the place where the grasping forceps were inserted and adhesion of ovary and uterus were detected in one sheep from group IV. No bilateral clinical lesions were detected in any animal.

Out of 90 ovaries, ovarian follicles were aspirated from 84 ovaries. The 6 remaining ovaries were not aspirated because of the lack of ovaraian folicles after hormonal stimulation. Total number of aspirated ovarian follicles was 204 (Table 2). From 204 aspirated follicles, 130 oocytes were recovered. The average recovery rate was 63.7%. The efficiency of sessions ranged from 58% to 75%.

Out of 130 oocytes, 112 were qualified for *in vitro* maturation (86.2%), including 46 (30.2%) in class I, 46 (35.4%) in class II and 16 (12.3%) in class III.

The remaining 18 oocytes (13.8%) were rejected due to cytoplasmic changes. An average, 4.5±2.54 ovarian follicles were aspirated from one sheep, 2.9±2.15 oocytes were recovered and 2.5±2.2 oocytes were accepted for maturation and fertilization.

The quality of oocytes obtained during the subsequent laparoscopic procedures was constantly decreasing, with the lowest quality of oocytes obtained during, the last 4th procedure. Oocytes of class I decreased from 43.2% in group I to 0.0% in group IV (Table 2). Oocytes of class II accounted for the average of 43.1% of all collected oocytes. In groups I, II and IV about 30-40% of class II oocytes were obtained, only in group III – 68.8%. However, differences between groups were statistically significant only in class I oocytes. There was increasing tendency of obtaining higher number of class III oocytes with higher number of procedures, from 0.0% in group I to 43.8% in group IV. The number of class IV oocytes was similar in groups I-III, only in group IV it was higher (up to 25%).

No correlation was found between subsequent recovery sessions and quantity recovered oocytes (Table 3) (0.06, p≤0.05), oocytes class II (r= 0.01, p≤0.05), oocytes class IV (r= 0.15, p≤0.05) and oocytes qualified for IVM (r = -0.05, 0.05). Statistically significant correlation coefficient between subsequent recovery sessions and quality recovered oocytes was only in class I and III oocytes and it was -0.33 (p≤0.05) and 0.48 (p≤0.05), respectively. The mean number of oocytes in I class was 30.8%. There was no correlation between oocytes recovery intervals and number of recovered oocytes (r = 0.16; p≤0.05). The mild negative correlation was found between time between recovery session and number of obtained class I oocytes (r = -0.43, p≤0.05) and between time of recovery and number of oocytes qualified for IVM (r= -0.31, p≤0.05). Positive mild correlation was found between time and number of obtained class IV oocytes (r = 0.33, p≤0.05). No correlation was found between time and number of obtained class II and III oocytes.

Discussion

During 40 years from its first application, laparoscopic method became the standard procedure that, in some cases, may replace surgical methods.

Based on the analysis of data in publications and previous own experience, an own method of laparoscopic oocyte retrieval was developed. General model of laparoscopic OPU in medium-sized farm animals, three or four trocars with diameter 5–10 mm and manipulators are installed. Endoscope is inserted through umbilicus or at the midline, grasping forceps and syringe or catheter for aspiration are put caudal and laterally to endoscope (Baldassarre et al. 1996, Kühholzer et al. 1997, Stangl et al. 1999, Teixeira et al. 2011).

We propose the new method of trocars insertion. Three trocars were inserted into abdominal cavity on isosceles triangle plan with arms of 15-20 cm, like it was described previously (Wieczorek et al. 2009). Optimal insertion of three trocars is scheduled as follows: left trocar – stiff endoscope with camera, medial one – s yringe or catheter for oocyte aspiration and right trocar – grasp forceps for ovary stabilization. This way of manipulators installation allows for easy access to aspirated follicles with possibility of oocytes recovery with wide view, covering abdominal organs and both pairs of grasping forceps as well as catheter. Ovary was stabilized by one grasp only. Although this stabilization is not so reliable and rigid as double ovary stabilization, it offers the possibility of convenient manipulation inside abdominal cavity and easy access to aspirated follicles (Wieczorek et al. 2005).

It is commonly accepted that laparoscopic surgery is minimally invasive and minimally traumatic metod, however in the literature there is no information concerning clinical observations as well as lesions after multiple surgical procedures in the same animals. That is why the special attention was given to the exact description of the type and dynamics of observed lesion. Only Stangl et al. (1999) reported clinical symptoms after laparoscopic OPU in sheep. The clinical complications, as adhesion between the ovary and infundibulum and adhesion between the uterus and omentum were observed in the study. One sheep out of 5 developed adhesion between uterus and omentum after 3rd session of OPU and became infertile. In our study, only in two animals unilaterally adhesion between the ovary and infundibulum were observed, after the second and the fourth session. Moreover, we did not observe adhesion between uterus and omentum. However, the additional clinical symptoms were observed in the present study. The unilateral adhesion of omentum and peritoneum was observed next to the location of the grasping forceps insertion in two sheeps, in one after the third procedure and in the another one after the fourth one. Unilateral adhesion of ovary and uterus was also observed in two sheeps, after the 3rd and the 4th sessions. The most common clinical symptoms were ovary bleeding and the scar at the place of needle insertion.

These clinically important lesions hindered or permanently prevented oocytes recovery. Adhesion of ovary and infundibulum permanently prevented oocytes recovery because of the lack of possibility of ovary isolation from infudibulum and finding oocytes in

ovary and their aspiration. The important fact is that in the case of ovary and infundibulum adhesion access to ovary and its stabilization were not hindered. In the case of ovary and uterus or omentum with peritoneum adhesion oocytes recovery was difficult and it was connected with the difficult access to ovary and difficult stabilization, whereas isolation of ovary from infudibulum, access to follicles with possibility of aspiration was not affected. The most commonly noticed clinical lesions, as bleeding and scar on the ovary surface after follicles punctures, had no influence on the way of follicles aspiration and the number and quality of recovered oocytes. The employed method and rare complications after operation, as clinical lesions mentioned above, had no influence on ovary functioning. In subsequent procedures, the increase of mean number of aspirated follicles and obtained oocytes for one donor was observed. However, the quality decrease of recovered oocytes was observed. The quality of oocytes obtained during the subsequent laparoscopic procedures was constantly decreasing, with the lowest quality of oocytes obtained during, the last 4th procedure. The factor that could possibly influence oocyte quality was short time period between subsequent laparoscopic procedures, what was showed by mean correlations between time of recovery and oocytes quality.

The quantity and quality of recovered oocytes was the indicator of the efficiency of the method. The main earlier established criteria had to be fulfilled to provide proper morphology of recovered oocytes and their ability for *in vitro* maturation. There are many different criteria that are used for oocyte assessment and classification, consisting of 2 to 6 degrees (Katska and Smorag 1984, Koeman et al. 2003, Katska-Ksiazkiewicz et al. 2007, Locatelli et al. 2012, Leisinger et al. 2014, Padilha et al. 2014). In our investigation, the four grade scale of oocytes assessment was used (Table 2). As the reasons for disqualification, the quality changes of cytoplasm were taken, independently of the quantity of granulosa cells. Koeman et al. (2003) and Locatelli et al. (2012) presented the similar scale, however they did not obtain such high efficiency of the method as it was obtained in the present investigation. The proposed modification had important impact on method efficiency and allowed for obtaining oocytes of about 25% higher quantity, without much influence on their quality.

According to the proposed four grade scale of oocytes assessment, for in vitro maturation only the oocytes with heterogenous cytoplasm were disqualified. In last years, much attention was paid to the possibility of in vitro maturation of denuded oocytes which are maturated with the supplement of granulosa cells. These studies were made in humans (Uyar et al. 2013) and different animal species, e.g.: mice (Ge et al. 2008),

cattle (Dias et al. 2014) pigs (Casillas et al. 2014), goats (Zhang et al. 2008) and sheep (Yasmin et al. 2015). In this case, it was decided to use reduced criteria assessment and qualification of recovered oocytes.

The proposed technique allows for efficient, repeatable and fast oocytes recovery with minimal trauma to living animals. The results obtained show that the proposed technique of laparoscopic oocyte recovery ensures the appropriate number of good quality oocytes, capable for *in vitro* maturation and which can be used in an IVM/IVF programme and cloning.

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