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Limitations of ultrasound guided follicular aspiration for analysis of ovarian follicular fluid in dairy cattle

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Abstract

The aim of this study was to evaluate the applicability of ovum pick-up equipment for follicular fluid collection from various follicular structures (experiment 1) and for recovery of follicular fluid for acid-base balance analysis (experiment 2). An ultrasound scanner equipped with a 5-MHz convex transducer was used for transvaginal ultrasound-guided follicular aspiration. A 17-gauge, 60-cm aspiration needle was connected with a shortened aspiration line. The fluid was aspirated manually into a 2 ml plastic syringe at a speed of approximately 0.2 ml/s. The success of aspiration was higher in ovarian cysts (100%) and single follicles larger than 13 mm (76.7%) compared to single follicles smaller than 12 mm (20%, $p < 0.001$). The success of aspiration of multiple follicles on day 4 (diameter of 7–9 mm) was higher (90.9%) compared to follicles on day 2 (diameter of 4-6 mm) (66.7%, $p < 0.05$) in experiment 1. The fluid from ovarian cysts > 25 mm in diameter was aspirated in a two-step procedure (samples 1 and 2) for the determination of pH, HCO_3, BE, pCO_2 and pO_2 (experiment 2). The indicators were compared between samples 1 and 2. Higher pO_2 as well as pH and lower pCO_2 in sample 1 compared to sample 2 showed insufficient anaerobic conditions during the first phase of the puncture in experiment 2. Our study brings for the first time the finding that the ovum pick-up equipment used in our experiments is suitable for the collection of follicular fluid only from larger follicular structures. The sampling of follicular fluid for acid-base balance assays requires the development of a special new device to prevent samples from coming into contact with air during aspiration.

Cow, heifer, collection of follicular fluid, ovum pick-up, follicles, cysts, acid-base indicators

Follicular fluid sampling has been performed for many years (Edwards 1974) for the evaluation of the oocyte microenvironment. *In vitro* collection is performed from ovaries obtained from slaughtered animals (Wise 1987; Leroy et al. 2004a; Orsi et al. 2005) and *in vivo* aspiration is performed in live animals as well (Ginther et al. 1997; Gérard et al. 2002; Jorritsma et al. 2003). The most frequently used method of *in vivo* aspiration is ultrasound guided transvaginal follicular aspiration (TVFA). This method was first described in 1988 as an ovum pick-up method (OPU) (Pieterse et al. 1988) and since then it has been used for oocyte collection in live cattle and horses (Pieterse et al. 1988; Brück et al. 1992). Various biochemical or endocrinological examinations of the follicular fluid collected by TVFA have been done (Vos et al. 1994; Kohram et al. 1998; Moallem et al. 1999; Landau et al. 2000; Walters et al. 2002; de Castro e Paula et al. 2008; Shehab-El-Deen et al. 2010) when dominant or preovulatory follicles larger than 10 mm in diameter were primarily chosen for follicular fluid collection *in vivo*. Recovery of the follicular fluid for acid-base balance (ABB) analysis from live animals has been rarely described (Berg et al. 2005; Cech et al. 2007) and technical aspects of the procedure were not evaluated. Although peripheral blood collection for ABB analysis is performed routinely and this procedure is easier than the recovery of follicular fluid, many preanalytical errors in blood sampling for this analysis have been described (Bandi 1981; James et al. 1997; Wu et al. 1997; Beaulieu et al. 1999).

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The aim of this study was to evaluate the applicability of transvaginal follicular aspiration by ovum pick-up equipment for the follicular fluid collection from single or multiple different sized follicular structures and for collection of follicular fluid for acid-base balance analysis in dairy cattle.

Materials and Methods

Ultrasound guided transvaginal follicular aspiration

Heifers were sedated by xylazine (10 mg *pro toto* i.m., Xylazin 2%, Bioveta a.s., Czech Republic) before the session. Transvaginal follicular aspiration was carried out after epidural anaesthesia (4 ml 2% lidocaine, Lidocaine 2%, Fatro, Italy), rectum evacuation and disinfection of the vulva and perineum. A real-time B-mode ultrasound scanner (SSD-500, Aloka, Japan), equipped with a 5-MHz convex transducer (UST-974-5, Aloka, Japan), was used to control aspiration. The transducer was mounted in a plastic handle (Eickemeyer 303922, Germany) with a stainless steel needle guide. A 17-gauge, 60-cm aspiration needle (V-OPAA-1760, Cook, Australia) was connected to a shortened Cook aspiration line (with a length of 20 cm) and an 18G needle (Terumo, Japan) was inserted into the end of the tubing. The total volume of the aspiration needle and aspiration line was 0.9 ml. Aspirations were performed manually into a 2 ml plastic syringe (Monovette®, Sarstedt, Germany) connected to an 18G needle at a speed of approximately 0.2 ml/s (negative pressure 50 mm Hg) (Plate I, Fig. 1).

Animals and treatment

Experiment 1: Collection of follicular fluid from variously sized follicular structures

Ultrasound guided transvaginal follicular aspiration of single follicular structures (dominant follicles and follicular ovarian cysts - Group A) or multiple follicles after follicle stimulating hormone (FSH) stimulation (Group B) was performed.

Group A

Eleven non-lactating Holstein cows (single aspiration) and 10 Holstein heifers (4-5 aspirations) housed at the University clinic were used for dominant follicle (DF) aspiration on day 7 of the oestrous cycle (oestrus = day 0). The diameters of the follicles were between 8 and 18 mm. In addition, aspirations were performed on 50 dairy cows bearing ovarian follicular cysts larger than 25 mm in diameter for therapeutic reasons and on 15 cystic dairy cows from commercial dairy farms specifically for the acid-base balance assay (experiment 2, see below). Overall, 55 single DFs and 65 ovarian cysts were aspirated and the success of aspirations of dominant follicles $\leq$ 12 mm as well as > 12 mm in diameter and ovarian cysts was evaluated.

Group B

Ten dairy heifers were stimulated (2–4 × at 2-month intervals) by FSH to obtain follicular fluid from a higher number of follicles at different developmental stages. Oestrus was induced by cloprostenol (500 µg i.m. pro toto, Oestrophan®, Bioveta a.s.). Seven days after this induction, the DFs were measured and punctured. Two days later (day 0), stimulation was initiated by 450 UI of FSH (Pluset®, Calier S.A., Spain) in eight decreasing doses (100, 75, 75, 50, 50, 25 and 25 UI) at 12 h intervals. Thirty three, 33 and 27 sessions were performed on days 2, 4 and 6, respectively. Two or three follicles were aspirated at each session and follicular fluid was pooled. The approximate diameters of the aspirated follicles were 4–6 mm, 7–9 mm and $\geq$ 10 mm on days 2, 4 and 6, respectively. The success of aspirations of follicles on days 2, 4 and 6 was evaluated.

Collection of at least 0.7 ml of follicular fluid without visible signs of the presence of blood (pink or red colour of the aspirated fluid) was considered a success.

Experiment 2: Collection of cystic fluid for acid-base balance analysis

Fifteen dairy cows housed at a commercial dairy farm were used for the collection of cyst fluid. Follicular ovarian cysts larger than 25 mm were diagnosed by ultrasonographic examination during regular visits to the dairy farms. Within the frame of one TVFA session the fluid was collected in two separate phases. One millilitre of cyst fluid was collected during the first phase of aspiration into the plastic syringe (flushing air from aspiration tubing) and a further 1 ml was obtained during the second phase of the aspiration after changing the syringe. The air bubbles were expulsed from the first syringe (sample 1) immediately after collection and the samples were inserted into a water bath with ice immediately after collection of the second sample (sample 2). Acid-base balance analysis was performed on a Rapidlab® 855 analyser (Bayer, USA) within 2 h of sampling. The values for pH, standard bicarbonate (HCO_3), base excess (BE), partial pressure of CO_2 (pCO_2) and O_2 (pO_2) were compared between samples 1 and 2.

Statistical analysis

The results were analysed by paired *t*-test and χ^2 test using Excel software.

Table 1. Success of fluid aspirations from single follicles and ovarian cysts

Diameter	Fluid aspiration		
	Total	Successful	%
Follicles ≤ 12 mm	25	5	20[a]
Follicles > 12 mm	30	23	76.7[b]
Cysts ≥ 25 mm	65	65	100[c]

Values with different superscripts are different ($p < 0.001$)

Table 2. Success of fluid aspirations from multiple stimulated follicles

Day	Fluid aspiration		
	Total	Successful	%
2	33	22	66.7[a]
4	33	30	90.9[b]
6	27	22	81.5[a,b]

Values with different superscripts are different ($p < 0.05$)

Table 3. Indicators of acid-base balance in cystic fluid - comparison between the first and the second phase of sampling

Sample	pH	pCO_2	pO_2	HCO_3 st.	BE
1 (n = 15)	7.341[a]	6.55[a]	18.22[a]	24.17	-0.36
2 (n = 15)	7.333[b]	6.86[b]	12.74[b]	24.42	-0.03

Values with different superscripts are different ($p < 0.05$)

Results

Experiment 1: Collection of follicular fluid from different sized follicular structures

Group A

Twenty-five follicles ≤ 12 mm, 30 follicles > 12 mm and 65 cysts > 25 mm in diameter were aspirated. The success of aspirations increased in accordance with the increase in the diameter of the aspirated structures (Table 1).

Group B

Thirty-three TVFA sessions on day 2 as well as on days 4 and 27 sessions on day 6 were performed. The success of aspiration was higher on day 4 compared to day 2 (Table 2). The total success of multiple follicles aspirations was 79.6% (74/93).

Experiment 2: Collection of cystic fluid for acid-base balance analysis

Each TVFA session was divided into two phases of aspiration (samples 1 and 2). In this way 30 samples from 15 ovarian cysts were obtained. The values of pH and pO_2 were higher in the first set of samples, while the values of CO_2 was higher in the second set of samples. The values of HCO_3 and BE did not differ (Table 3).

Discussion

Only a few studies have evaluated the follicular fluid aspiration system in comparison with the recovery rate of follicular fluid under *in vivo* condition. These studies have described aspirations from single follicles of various sizes 6–12 mm (Ginther et al. 1997), more than 8 mm (Leroy et el. 2004b) and more than 10 mm (Kendrick et al. 1999) using needles of various diameters 17G (Kendrick et el. 1999), 18G (Jorritsma et al. 2003), 20G (de Castro e Paula et al. 2008), 21G (Leroy et al. 2004b) and 25G (Ginther et al. 1997). Unsuccessful aspirations were the result of blood contamination of the follicular fluid (Ginther et al. 1997; Jorritsma et al. 2003), difficult manipulation of the ovaries (Ginther et al. 1997) or by the rupture of follicles (de Castro e Paula et al. 2008). However, a direct description of aspiration success was not reported frequently. A high aspiration success 86% (113/132) has been reported in follicles with a diameter of 6–12 mm in heifers using the thinnest needles (25G, diameter 0.52 mm) even without any adverse effects on the post-sampling condition of the aspirated follicle (Ginther et al. 1997). This success rate was better than in our study; however, the average amount of collected follicular fluid was only 20 µl, compared to 0.7 ml follicular fluid obtained in our study. Furthermore, we used very thick needles (17G). The success of aspiration of preovulatory follicles using similar equipment with a 20G needles was 78.6% (22/28)

(de Castro e Paula et al. 2008), which is comparable with our results for large follicle aspiration (76.7%). In our study, the success of aspiration increased in accordance with the increase in the diameter of the aspirated follicular structures, and ultimately resulted in the recovery rate of 100% in ovarian cysts. Unsuccessful aspirations in our study were caused by insufficient volume (< 0.7 ml) or blood contamination. We assumed that a large needle diameter (17G, outer diameter of 1.5 mm) as well as a large volume of aspiration tubing (0.9 ml) were the reasons for any unsuccessful aspirations in our study. Standard OPU equipment with a 17G aspiration needle was suitable only for follicular fluid collection from larger, single follicular structures > 12 mm in diameter, because the succcess of aspiration was significantly higher in these follicles compared to smaller ones.

The first study concerning follicular fluid sampling after FSH stimulation reached a total recovery rate 81.2% (Vos et al. 1994). In this study, the authors aspirated follicles with diameters between 8-15 mm twice in three groups of cows. Recovery rates of the first collection tended to be higher compared to the second collection 82.8% vs. 76.5%, 85.3% vs. 80.9% and 89.7% vs. 80.6% (Vos et al. 1994). We observed a similar success rate for stimulated aspirations in our study 79.6%) (74/93). However, the lowest recovery rates at the first aspiration of stimulated follicles on day 2 (66.7%) occurred when the smallest follicles, about 5 mm in diameter were aspirated. The dynamics in aspiration success were similar to the above mentioned study 90.9% on day 4 vs. 81.5% on day 6, although the largest follicles were punctured on day 6. This could have been caused by a more difficult search for follicles in the ovaries after two repeated aspirations. In our study, aspiration of multiple follicles larger than 8 mm in diameter was easy, but obtaining a sufficient amount of follicular fluid from smaller multiple follicles was difficult. It is usually impossible to aspirate more than 2–3 small follicles with a 17G aspiration needle without blood contamination. Our equipment was suitable for aspiration of multiple follicles larger than 7 mm in diameter. We assume that an ultrasound transducer with a frequency higher than 5 MHz and thinner aspiration needles could be more suitable for aspiration of smaller developing follicles as well as for repeated aspirations.

Aspiration of follicular fluid for the analysis of acid-base balance assay has rarely been done in cattle. We have found only one study that performed sampling of bovine follicular fluid from ovaries after slaughter (Redding et al. 2006) and one study employing TVFA for this reason (Berg et al. 2005). A few articles in human medicine have described the concentration of carbon dioxide and pH in follicular fluid (Daya 1988; Imoedemhe et al. 1993) and oxygen concentration has been described in bovine follicular fluid after TVFA of preovulatory follicles (de Castro e Paula et al. 2008). Preanalytical changes of samples taken for acid-base analysis due to errors in manipulation and incorrect storage of samples can occur easily. Blood samples should be obtained anaerobically and collection tubes should be completelly filled, otherwise CO_2 can escape from the serum to the partial vacuum above (Bandi 1981; Constable 2008). The underfilling of blood collection tubes results in a false decrease of CO_2 concentration in serum and can result in a false diagnosis of metabolic acidosis in dogs and cats (James et al. 1997). Acid-base indicators can also be changed by storage temperature or type of syringe (plastic or glass) (Wu et al. 1997; Beaulieu et al. 1999). Because similar errors should be expected during and after collection of follicular fluid (Redding et al. 2006), the requirements for anaerobic follicular fluid collection have been reported (Berg et al. 2005; Redding et al. 2006). However, changes in the quality of follicular fluid can occur even before aspiration due to increasing exposure to intraperitoneal CO_2 during artificial pneumoperitoneum with laparoscopical procedures for follicular aspiration. This has been associated with a decrease in the pH of follicular fluid (Daya 1988; Imoedemhe et al. 1993).

In experiment 2 of our study, we assumed that the first set of samples was influenced by air in the empty tubing line, and the second set of samples was collected under strictly anaerobic conditions after flushing the tubes and needle during the first phase of aspiration

by cystic fluid. We have proven that O_2 diffused into and CO_2 escaped from the first samples during the first phase of aspiration. This was based on the higher O_2 concentration and the lower CO_2 concentration in the first samples compared to the second samples. In accordance with these changes, we found higher pH values in the first set of samples compared to the second set of samples. Similar changes in the first part of the collected follicular fluid have been described by Redding et al. (2006). However, the volume of the aspiration tubes and the concentration of CO_2 were not described and follicular fluid was obtained from slaughtered cows in this study. The flushing of aspiration tubes by the first part of the sample used in our study was not feasible for the aspiration of smaller individual follicles. Application of thinner needles with extra thin flexible plastic tubes can minimise the changes (Shehab-El-Deen et al. 2010) but this does not completely solve the problem.

On the basis of our results we concluded that standard OPU equipment with a 17G aspiration needle is not suitable for the collection of follicular fluid for acid-base balance assays. The sampling of follicular fluid using TVFA requires the development of a special new device preventing the samples from coming into contact with air during aspiration.

Limity transvaginální sonografické aspirace folikulů pro analýzu folikulární tekutiny u mléčného skotu

Cílem práce bylo zhodnotit použitelnost zařízení pro transvaginální sonografickou aspiraci oocytů k odběru folikulární tekutiny u mléčného skotu z folikulů různých velikostí a pro vyšetření acidobazické rovnováhy. K odběru byl použit sonograf s 5 MHz konvexní sondou ve speciálním držáku se zařízením k punkci folikulů aspiračními jehlami 17G délky 60 cm. Podtlaková aspirační souprava byla zkrácena (20 cm) a po napojení na aspirační jehlu celkový objem aspiračního systému činil 0,9 ml. Folikulární tekutina byla nasávána manuálně do plastové injekční stříkačky rychlostí přibližně 0,2 ml/s. Byla hodnocena úspěšnost aspirací jednotlivých folikulů s průměry ≤ 12 mm, > 12 mm a ovariálních cyst > 25 mm a rovněž úspěšnost aspirací multipních folikulů v den 2, 4, 6 po zahájení stimulace pomocí FSH (experiment 1). Dále byla získávána tekutina ovariálních cyst o průměru > 25 mm dvoufázovou aspirací (vzorky 1 a 2) pro stanovení pH, HCO_3, BE, pCO_2 a pO_2 a tyto parametry byly srovnány mezi vzorky 1 a 2 (experiment 2). V experimentu 1 byla úspěšnost aspirace vyšší u větších jednotlivých (> 13 mm) nebo multipních (7–9 mm) folikulů a cyst ve srovnání s ostatními folikulárními útvary. V experimentu 2 vyšší pO_2 i pH a nižší pCO_2 u vzorků 1 prokázalo nedostatečné anaerobní prostředí během první fáze punkce. Z výsledků uzavíráme, že zařízení pro transvaginální sonografickou aspiraci oocytů je vhodné pouze pro získávání folikulární tekutiny z větších folikulárních útvarů. Odběr folikulární tekutiny pro stanovení acidobazické rovnováhy vyžaduje vývoj nového zařízení, které by zamezilo kontaktu vzorku se vzduchem během aspirace.

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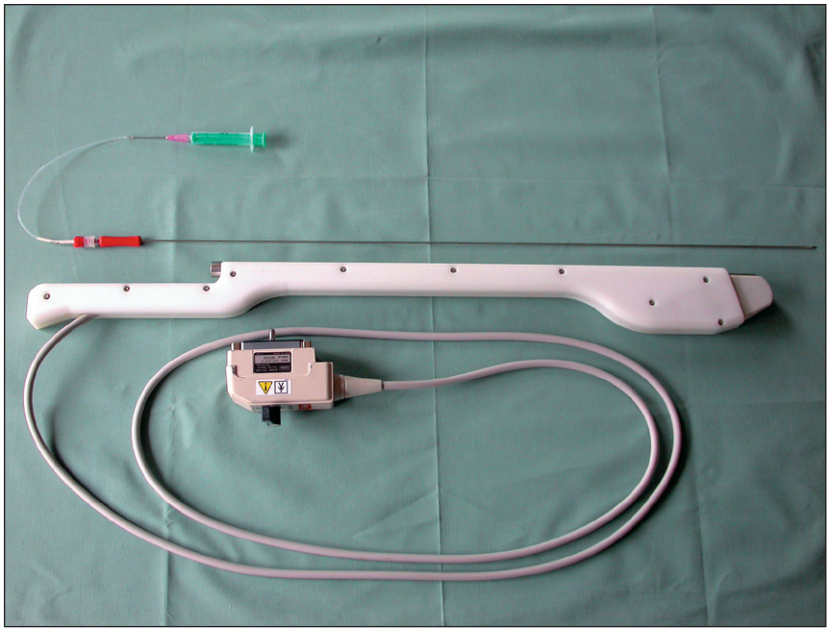

Fig. 1. Equipment for follicular aspiration (aspiration needle, aspiration line, 18G needle, syringe).