How to Collect Equine Oocytes by Transvaginal Ultrasound-Guided Follicular Aspiration

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1. Introduction

Assisted reproductive techniques such as oocyte transfer and intracytoplasmic sperm injection (ICSI) can be used clinically to produce foals from mares with reproductive abnormalities that prevent normal breeding or production of embryos.[1–3] ICSI can also be used to produce foals from stallions with poor fertility or from semen with limited quantity.[4–6] Both of these procedures require the collection of oocytes. Ultrasound-guided transvaginal oocyte aspiration (TVA) can be used by the practitioner to recover maturing oocytes from dominant gonadotropin-stimulated follicles (DSF) or immature oocytes from smaller or subordinate follicles (IMM) for use in these techniques. DSF oocytes are generally required for oocyte transfer, whereas both DSF and IMM oocytes are suitable for ICSI. Once collected, the oocytes can be used on-site or shipped to a referral laboratory for embryo production.

2. Materials and Methods

Equipment required for TVA includes the following:

(a) Transvaginal ultrasound probe with needle guide,[a] preferably with a micro-convex ultrasound probe

(b) 60-cm, double-lumen, 12-gauge oocyte aspiration needle[b]

(c) Aspiration pump with a vacuum/pressure relief valve[c] capable of maintaining a regulated negative pressure of 150 mm Hg[d]

(d) Collection bottle(s)[e] and water bath[e] set to 37°C; 250-mL bottles are a convenient size

(e) 150-mm sterile petri dishes[f]

(f) Warming tray[g] set to 37°C

(g) Dissecting microscope with transmitted light source as used for embryo search and identification

(h) 0.25-mL semen straws

(i) Pipettor and pipettes

(j) Embryo filter[h]

(k) Complete embryo flush medium[i] with 5 IU heparin[j]/mL

(l) Controlled flushing set[k]

(m) 20-mL all-plastic syringe[l]

(n) Glutaraldehyde[m] for sterilization of ultrasound probe and needle guide

Equipment Setup

A 2-L bag of complete embryo flush medium with 5 IU heparin/mL that has been pre-warmed to 37°C is attached to the controlled flush set and is suspended from an IV stand. A 20-mL, all-plastic syringe is attached to the female connector of the controlled flush set and the male connector of the controlled flush set is attached to infusion tubing of the aspi-

NOTES

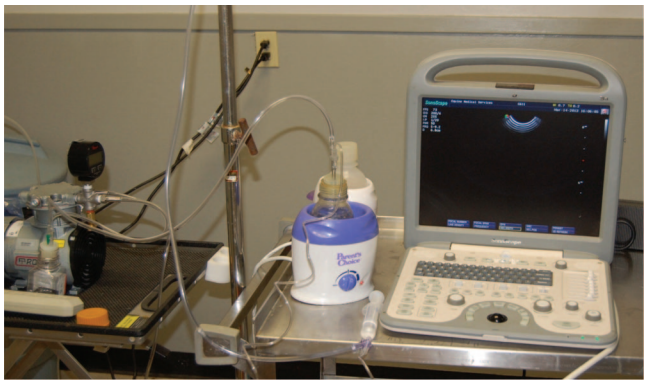

Fig. 1. Simple aspiration setup with the use of thermostatically controlled baby-bottle warmers as water baths.

ration needle. The controlled flushing set has an automatic three-way valve that allows an assistant to alternatively rapidly fill the syringe and then infuse flush medium into the follicle by simply moving the syringe plunger. Tubing from the aspiration port of the needle (the center lumen) is attached to the collection bottle placed in a 37°C water bath. The vacuum pump, regulated to −150 mm Hg, supplies the vacuum through the collection bottle (Fig. 1). The needle is inserted into the needle guide, with care taken to avoid contamination and to prevent dulling the needle.

Mare Preparation

DSF Oocytes

Oocytes are firmly adhered to the wall of the follicle. After the luteinizing hormone (LH) surge, as ovulation approaches, the attachment loosens in dominant follicles. Stimulation of the LH surge with an ovulatory agent such as deslorelin acetate[n] allows a timed collection of the oocyte 24 to 36 hours later.[7,8] This timing allows collection of an oocyte that has become easier to flush and has also started maturation and will not need additional hormonal stimulation. A typical mare is administered 1.8 mg of deslorelin acetate after detection of a 35-mm actively growing follicle during estrus in association with endometrial edema, but this will vary with breed, individual, and time of year.

IMM Oocytes

Immature oocytes can be collected from small follicles at any stage of the estrous cycle or from subordinate follicles during estrus. Equine oocytes are especially firmly adhered to the follicle wall in a broad-based hillock of cumulus cells. Considerable intra-follicular turbulence is necessary to dislodge the oocyte to allow it to be flushed out of the follicle. The greatest productive oocyte yield will generally be from follicles of 10 to 20 mm diameter, because it is difficult to create the necessary turbulence in larger follicles, and oocytes in smaller follicles are often not meiotically competent. Regular examination can allow scheduling of the follicular aspiration when the largest population of follicles in the appropriate size range are present.

Follicular Aspiration

All Mares

Mares are restrained in stocks and tranquilized to effect with approximately 0 01 mg/kg detomidine HCl[o] or 0.66 mg/kg xylazine[p] intravenously. *N*-butylscopalammonium bromide[q] (0.9 mg/kg IV) is administered to encourage rectal relaxation and to prevent peristaltic waves from interfering with trans-rectal ovarian manipulation. The tail is tied up, the rectum is evacuated, and the vulva and perineum is cleaned. With a sleeved hand, approximately 2 oz of obstetrical lubricant is distributed over the face of the ultrasound probe and holder with the needle guide. A thin rectal sleeve is placed and then stretched over the probe and holder to prevent vaginal mucus and debris from being introduced into the needle guide channel. Obstetrical lube is applied to the exterior of this sleeve and the probe is guided into the vagina and placed against the dorsal anterior vaginal wall lateral to the cervix. The sleeved hand is withdrawn from the vagina and introduced into the rectum. The ovary is grasped and guided to the dorsal vaginal wall next to the transducer. This requires manipulating the ovary and lifting it up over the uterus and is best accomplished by grasping the ovarian suspensory ligament between two fingers. The ovary and probe are aligned in such a manner that the follicle(s) to be aspirated are adjacent to and aligned with the needle guide. Penetration of the vaginal wall, ovary, and follicle with the needle is facilitated by firm pressure of the ultrasound probe against the vaginal wall opposed by firm pressure of the ovary against the peritoneal surface of the vaginal wall. The needle is advanced to the center of the follicle, and the aspiration pump is started (Fig. 2).

Initially, the practitioner may find it easiest to simply manipulate the probe and the ovary while having an assistant advance the needle into the follicle. This works well with dominant follicles, but smaller follicles are more efficiently aspirated when the operator handles the needle as well as the probe and ovary. This allows all to move or be stabilized in synchrony with the timing of needle puncture; which is especially important if the animal is moving. The probe handle is held in the palm of the hand with forward pressure maintained by the heel of the hand. The fingers are then free to advance and manipulate the aspiration needle (Fig. 3).

DSF Oocytes

Once penetrated, the follicle is completely evacuated and then re-filled as rapidly as possible while the

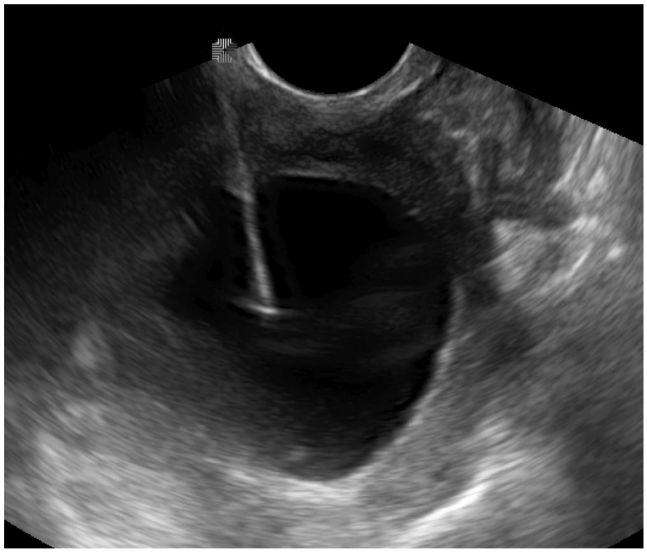

Fig. 2. DSF with needle advanced to center of follicle.

pump remains running. The speed of fluid refilling the follicle encourages the turbulence that is responsible for dislodging the cumulus oocyte complex from the follicle wall. When possible, the operator ballots the emptying follicle to increase turbulence during the evacuating phase as well. The follicle is sequentially filled and evacuated approximately 10 times as the volume in the collection bottle allows.

Oocytes from dominant stimulated follicles are quite temperature sensitive. Small decreases in temperature for even a short period can cause depolymerization of the meiotic spindle and subsequent fertilization abnormalities.[9,10] Equipment, labware, and solutions that the oocyte will come in contact with should be kept as close to body temperature as possible to prevent spindle damage. The flush solution should be pre-warmed, the collection bottles maintained at body temperature, and petri dishes for searching should be kept on a warming tray (Fig. 4).

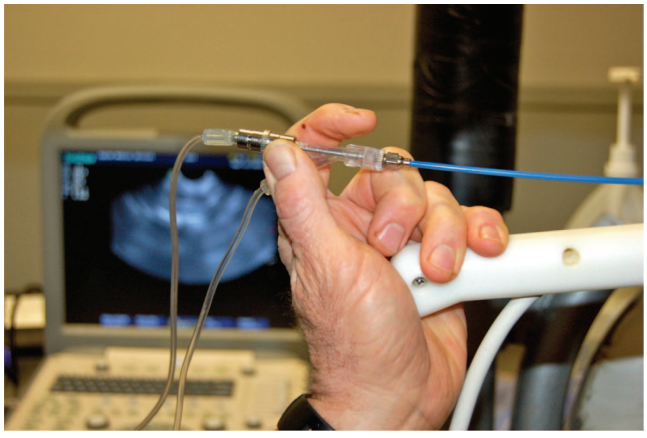

Fig. 3. Probe handle is held with the palm of the hand, allowing the thumb and first two fingers to manipulate the needle.

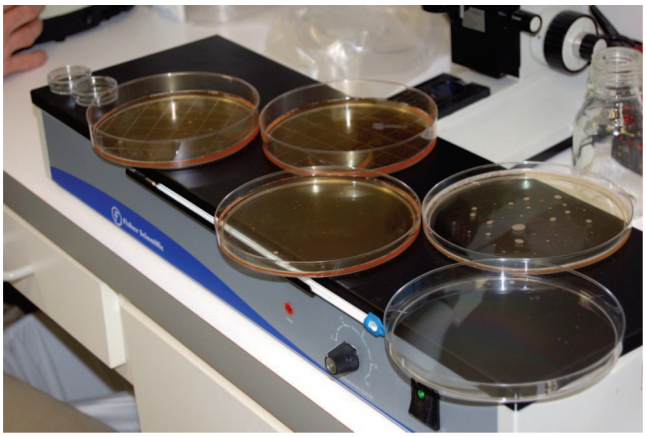

Fig. 4. Fluid from DSF aspiration distributed in 150-mm petri dishes on a warming tray.

IMM Oocytes

Once penetrated, the follicle is completely evacuated, then refilled as quickly as possible while the pump remains running, creating as much intra-follicular turbulence as possible. The follicle wall is lightly scraped with the needle by gently manipulating the probe and the ovary during aspiration. The needle can also be rotated to assist in freeing the oocyte from the follicle wall.

Generally, several small follicles can be aspirated with a single needle puncture. The ovary and needle guide can be manipulated to allow the needle to be advanced through the ovarian stroma into successive follicles. The aspiration pump is kept running as the needle is advanced from one follicle to the next but is turned off when the needle is withdrawn from the ovary to prepare for another puncture, or at the end of the procedure.

IMM oocytes are not as temperature-sensitive as DSF oocytes, but temperature shock is avoided to decrease cellular stress.

Search and Identification of Oocytes

DSF Oocytes

Fluid recovered from stimulated dominant follicles is usually bloody; to effectively search for a cumulus oocyte complex (COC), the fluid is distributed into 150-mm petri dishes on a warming tray. Blood is generally present from the aspirated fluid of dominant follicles that have responded to gonadotropins; therefore bloody aspirations are more likely to yield a maturing oocyte. Clear recovered fluid is more commonly recovered from follicles that have not responded to gonadotropins, although this is not always the case.

The petri dishes are individually searched for the COC with the use of the dissecting microscope at

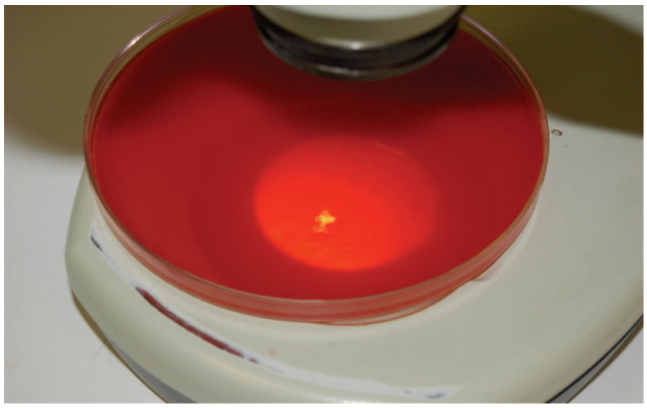

Fig. 5. Clear COC from dominant gonadotropin-stimulated follicles (DSF) aspiration in search dish.

×10 to ×25 magnification. The COC often appears as a large, clear, fluffy mass of cells containing the oocyte and is easy to identify in the bloody fluid (Fig. 5). If it is not readily found, all cell masses should be closely examined. The oocyte appears as a partially dark sphere surrounded by the corona radiata in the center of the clear cumulus mass (Fig. 6). The cytoplasm of the meiotically competent oocyte generally appears heterogeneous in nature; it has dark areas and light areas caused by accumulations of lipids. Handling the COC, because of its large size, is performed with a 0.25-cc semen straw or a fire-polished glass pipette of similar diameter (1.5 mm).

IMM Oocytes

Recovered fluid from small follicles is variably contaminated by blood. Oocytes are located by filtering the fluid through an embryo collection filter and then further clarifying by passing more flush medium through the filter. The clarified fluid is rinsed into a 150-mm petri dish, which is then searched for

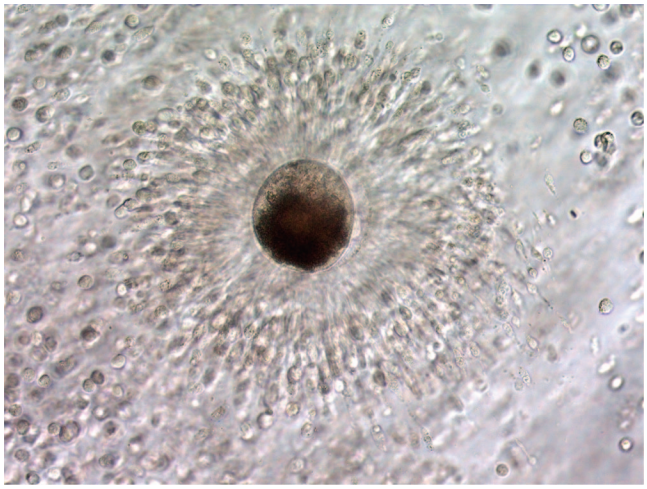

Fig. 6. COC from DSF aspiration.

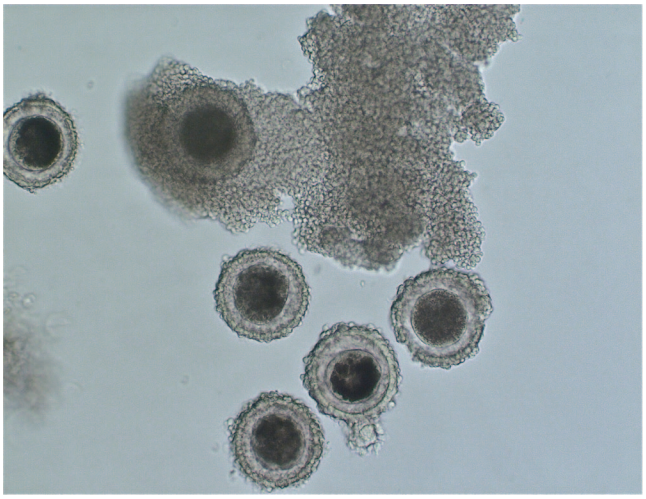

Fig. 7. Compact COC from IMM follicle aspiration.

oocytes. The COC of the IMM is usually quite dense, although occasionally an expanded cumulus is noted, but the expansion is not usually as dramatic as those of the DSF. Oocytes are usually found individually in the dish, or clumped with cellular debris, surrounded by only a few layers of cumulus cells or contained in a small, dense, relatively flat mass of cumulus cells (Fig. 7). These small COC can be handled effectively with a pipettor and 10-μL tips.

3. Results

Oocyte recovery rates are generally approximately 75% or more for DSF oocytes[11,12] and 50% to 60% for IMM oocytes,[3,13] although in the authors' experience, selection of follicles between 10 and 20 mm in diameter can increase percent recovery of IMM oocytes. Mares and breeds prone to multiple dominant follicles and larger numbers of subordinate follicles provide an opportunity for larger numbers of oocytes to be recovered per aspiration session, whereas older mares tend to have fewer subordinate follicles and will generally yield fewer IMM oocytes per aspiration session.

During 2012, in the authors' clinical practice, 223 aspiration sessions performed on 42 client-owned mares, between the ages of 12 and 26 years, yielded 126 intact DSF oocytes and 829 IMM oocytes. Of the 829 IMM oocytes that were collected, 611 matured to metaphase II (73.7%) as evidenced by extrusion of a first polar body. ICSI was performed on intact DSF oocytes and metaphase II IMM oocytes, resulting in 126 blastocysts; 44 of the blastocysts were produced from DSF oocytes (0.35 blastocyst per oocyte) and 82 from IMM oocytes (0.13 blastocyst per injected oocyte). Blastocysts (n = 106) were transferred non-surgically into recipient mares, resulting in 83 pregnancies (78.3%) at 14 days. Fifteen pregnancies were lost by 30 days

(18.1% embryo loss), and 20 of the blastocysts produced were cryopreserved.

4. Discussion

Ultrasound-guided transvaginal follicular aspiration is a useful technique for collection of oocytes for advanced reproductive techniques. The ability of the practitioner to successfully retrieve oocytes from mares will allow more owners access to these reproductive techniques. TVA is not a complex procedure, but most individuals require some practice to become proficient at the technique, and, to some extent, increased practice will continue to increase proficiency. One should endeavor to obtain this practice, preferably under the guidance of someone already experienced, and master the technique before attempting TVA on a client's animal.

TVA has been shown to be a safe procedure; rare but reported negative effects include internal hemorrhage,[14] adhesion development,[15] and ovarian abscessation.[16] The authors have only noted one case of ovarian abscessation in an estimated 1000+ clinical, research, and teaching TVA sessions. Repeated aspiration sessions have also shown no negative impact on cyclicity[15] or future fertility[17–19] of mares studied. This does not mean that the technique should be taken lightly; attention should be paid to proper technique, restraint, and aseptic technique. The risk of rectal tears is present with any rectal palpation procedure and ovarian manipulation.[16] Many of the mares presented for oocyte collection have chronic endometritis or pyometra, contributing to the likelihood of vaginal contamination that could be carried into the abdomen through needle puncture. Prophylactic treatment with antibiotics has been reported,[20] and the administration of ampicillin and gentamicin 10 minutes before TVA has been shown to have no adverse effect on blastocyst formation rates after ICSI,[21] although the administration of antibiotics may be considered unnecessary by some because of the relative infrequency of TVA-associated infection.

Restraint and analgesia for TVA are easier to attain in some mares than others. A dose of 300 mg of xylazine administered intravenously will provide adequate sedation for most pluriparous mares, but mares that are more fractious may require an increased dose of xylazine or detomidine or use of a muzzle twitch. Mares with DSF that are aspirated near ovulation may be sensitive to manipulation, requiring additional analgesia, as are mares in which significant tension on the ovarian suspensory ligament is necessary to position the ovary for TVA. Pain caused by tension of the suspensory ligament may be alleviated somewhat by the administration of flunixin meglumine before TVA, whereas ovarian sensitivity is best handled by increased dosage of detomidine or xylazine.

The TVA technique requires the ability of the operator to manipulate the ovary back to the cranial surface of the vaginal wall. This can be difficult in mares with short ovarian suspensory ligaments and short vaginal vaults, which is often the case in maiden mares. Micro-convex ultrasound probes better facilitate aspiration in these mares compared with linear-array probes because the orientation of the probe in the needle guide allows the ovary to be in front of the micro-convex probe, whereas it must be pulled farther caudally to be on top of the linear probe (Fig. 8).

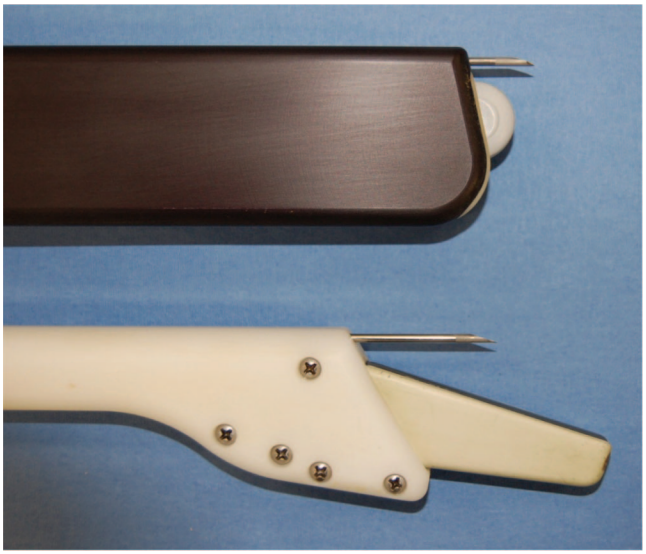

Fig. 8. Demonstration of the orientation of the needle exiting both the micro-convex probe and linear probe needle guides.

Scheduling of an aspiration session depends somewhat on the intended type of recovered oocyte. TVA for recovery of DSF oocytes or DSF with IMM oocytes is generally scheduled on the basis of ultrasound examinations and progression of the donor mare's estrous cycle, although aspiration on a 14-day interval without ultrasound examination[21] was found to be effective for both DSF and IMM oocytes. Aspirations performed for collection of only IMM oocytes can generally be performed at the convenience of the mare manager and veterinarian, although periodic ultrasound examinations can help select a time with higher follicle numbers. TVA performed at an interval less than every 10 to 11 days may deplete follicle numbers, resulting in fewer follicles aspirated per session.[19,20]

Maintenance of aspiration equipment is paramount for mare safety as well as the effectiveness of the procedure. Sterilization of the aspiration needles is somewhat problematic because of their size as well as the combination of metal and plastic parts. Gas sterilization can be used, but a significant de-gassing time period is necessary because of the extreme sensitivity of oocytes to toxins. Dry-heat sterilization has been an effective alternative in the authors' practice. Heating in a common household oven at 170° F for 2 hours or longer sterilizes the needle and tubing as long as it is com-

pletely dry, although the authors typically "bake" them overnight. Needles will dull with repeated uses, so replacing or re-sharpening is necessary. The authors sharpen needles with an ultra-fine ceramic stone[r] under direct visualization through a dissecting microscope as necessary or after three to four aspiration sessions. Sterilization of the ultrasound probe and needle guide is accomplished through immersion in glutaraldehyde for 20 minutes. The probe and guide assembly are then thoroughly rinsed with distilled or deionized water and allowed to air-dry.

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[a]Needle guide, Minitube of America, Inc, Verona, WI 53593.
[b]60-cm, double-lumen, 12-gauge oocyte aspiration needle, Minitube of America, Inc, Verona, WI 53593.
[c]CDI Control Devices Valve, Relief, ¼-inch, 5z763, Grainger, Lake Forest, IL 60045.
[d]Gast Roc-R Vacuum Pump 1/8 hp model 5z669, Grainger, Lake Forest, IL 60045.
[e]Kimax, Kimble Chase Life Science and Research Products, LLC, Rockwood, TN 37854.
[f]Corning, Inc, Corning, NY 14831.
[g]Fisher Scientific, Waltham, MA 02454.
[h]Embryo transfer low volume filter, Veterinary Concepts, Inc, Spring Valley, WI 54767.
[i]Vigro Complete Flush, Bioniche Animal Health, Belleville, Ontario K8N5J2, Canada.
[j]Heparin sodium injection, Sagent Pharmaceuticals, Schaumburg, IL 60195.
[k]Controlled flushing set CFS36, Mila International, Inc, Erlanger, KY 41018.
[l]20-mL Norm-Ject. Restec Corp, Bellefonte, PA 16823.
[m]CidexPlus, Johnson and Johnson, Irvine, CA 92618.
[n]SucroMate, Thorn BioScience, LLC, Louisville, KY 40204.
[o]Dormosedan, Orion Corp, Espoo, Finland; Zoetis, Madison, NJ 07940.
[p]AnaSed, Lloyd Laboratories, Shenandoah, IA 51601.
[q]Buscopan, Boehringer Ingelheim Vetmidica, Inc, St. Joseph, MO 64506.
[r]Spyderco, Inc, Golden, CO 80403.