Absence of pregnancies following transfer of manually collapsed equine expanded blastocysts vitrified and warmed using a modified commercial system

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Abstract

The availability of suitable, estrous cycle-synchronized embryo recipient mares can be a limiting factor for practitioners performing embryo transfer. Consequently, methods for vitrification and long-term storage of equine expanded blastocysts were developed. The most successful of these methods require micromanipulator-facilitated blastocoele collapse and subsequent dehydration/rehydration of embryos during vitrification and warming. However, difficulty and expense of these techniques limit blastocyst vitrification/warming to tertiary referral centers staffed by highly trained individuals. A simplified method that would make the process accessible to field practitioners is highly desirable. In conversations with practitioners, we learned that, in the absence of a suitable recipient mare, some field veterinarians attempt manual blastocoele collapse using a small gauge hypodermic needle prior to embryo vitrification with a commercial 'kit'. Pregnancy outcomes following warming and transfer of these embryos are not known. We duplicated this field technique in a controlled setting and determined subsequent pregnancy rates. To further mimic the scenario that might be encountered in general practice, all embryo manipulations were performed by veterinarians familiar with routine handling of fresh embryos, but with little to no experience with embryo collapse or vitrification. Twelve Grade I, large ($\geq$ 290 μm) blastocysts were recovered from donor mares following routine embryo flush done 8 days after ovulation. Embryos were manually collapsed with a 25 gauge hypodermic needle and vitrified using a commercial kit. Six embryos were lost or damaged during collapse, vitrification or warming. The remaining 6 embryos were warmed and transferred to recipient mares by a veterinarian highly experienced with transcervical embryo transfer. No pregnancies resulted; therefore, we suggest that this simplified technique, at least when performed by inexperienced operators, is not suitable for clinical use.

Keywords: Equine, embryo, blastocyst, vitrification

Introduction

Embryo Transfer (ET) is an assisted reproductive technique used when performance or health issues prevent a donor mare from carrying or maintaining a pregnancy. The technique involves breeding a donor mare and recovering the embryo by flushing the donor mare's uterus on days 7 or 8 after ovulation, when it has typically reached the blastocyst stage and is ~ 300 μm or more in diameter. The embryo is then transferred transcervically into a recipient mare whose estrous cycle has been synchronized with that of the donor. Pregnancy rates (PRs) following transcervical transfer of fresh embryos range from 74 - 81%.[1,2]

Expense and labor to maintain a sufficient number of fertile, estrous cycle-synchronized, recipient mares are major limiting factors to clinical ET. This is particularly relevant to private practitioners who may not have access to large recipient herds. When multiple donor mares ovulate on the same day and/or when a donor mare ovulates more than one follicle on a cycle, there may be an insufficient number of estrous cycle-synchronized recipient mares to accommodate fresh transfer of each embryo. In this regard, the ability to successfully preserve equine embryos would greatly facilitate equine ET by allowing embryos to be stored indefinitely until a suitable recipient is available.

Preservation of smaller equine embryos (250 - 400 μm) by vitrification can be very successful, with some reporting up to a 75% per transfer PR following warming.[3-9] In particular, pregnancies were reported when a simple, closed vitrification technique was performed on smaller embryos (morula or early blastocyst stage and < 250 μm).[10] This method is now available in a commercial 'kit' that allows for vitrification with minimal laboratory equipment and minimal training. However, to obtain these smaller

embryos, the embryo flush must be carefully timed and performed on day 6 to 6.5 after ovulation or 8 days after properly timed administration of human chorionic gonadotropin (hCG). When a flush is scheduled based on day of ovulation, embryo recovery rates can be significantly lower (23 - 66%) than those reported for days 7 or 8 flushes (49 - 82%).[10,11] Because of the careful timing required for these early flushes, because the embryo is smaller and more difficult to identify, and because of the potential for lower recovery rates, in clinical practice, the vast majority of uterine flushes for embryo recovery are performed on days 7 or 8 after ovulation. However, vitrification and warming of intact, expanded blastocysts (> 400 μm) commonly obtained on days 7 or 8 flushes results in poor success following embryo transfer.[4,12] Until per transfer PRs following vitrification and warming of days 7 or 8 expanded blastocysts approach those achieved following fresh embryo transfer, embryo vitrification will continue to be of limited use in equine clinical practice.

It was suggested that the embryonic capsule, together with the large size of the blastocoele cavity, may limit penetration of cryoprotectants into expanded equine blastocysts and therefore may contribute to the low survival of these embryos following vitrification and warming.[4,13,14] In this regard, per transfer PRs ranging from 46 - 86% were reported when expanded blastocysts are punctured and/or collapsed prior to vitrification.[13,15,16] Highest PRs were achieved when micromanipulator-facilitated puncture and collapse of the blastocoele was combined with stepwise dehydration in increasing concentrations of ethylene glycol and galactose or sucrose, either by immersion or by direct microinjection of the solution into the collapsed blastocoele, and subsequent vitrification using an open vitrification system with microloader tips.[13,15] Warming was also performed in an open system and included stepwise rehydration in decreasing concentrations of galactose or sucrose, with or without subsequent embryo culture.[13,15] However, technical difficulties and costs involved with these techniques have limited their use to specialty institutions with access to advanced laboratory equipment and individuals with considerable expertise in the use of this equipment.

The objective was to develop an alternative method for blastocoele collapse and vitrification that did not require highly trained personnel or the use of a micromanipulator. We combined a manual method of blastocoele collapse with a simple, closed, commercial equine embryo vitrification and warming system. After warming, each embryo was rehydrated using a stepwise system that had previously been reported to result in pregnancies following transfer of collapsed, vitrified expanded blastocysts.[13] Additionally, all advanced embryo manipulations (embryo collapse, vitrification and warming) were performed by veterinarians who commonly worked with fresh embryos, but who had no prior experience with embryo collapse or vitrification, similar to the situations of many private practitioners working in equine theriogenology. In simplifying these techniques, and in having them performed by individuals with no prior experience with more advanced embryo manipulations, our goal was to develop an approach to blastocyst vitrification that could readily be adapted to clinical practice and so render ET more cost-effective for the client and more accessible to the equine practitioner.

Materials and methods

Animal care and oversight

All procedures conducted on live animals were approved by the University of Pennsylvania's Institutional Animal Care and Use Committee. Animals were maintained on pasture with supplemental hay as needed.

Embryo donor mares

Six light breed mares, ages 7 - 15 years, were used for artificial insemination and subsequent embryo recovery. All mares selected as embryo donors were known fertile mares that had been bred with fresh semen and maintained a pregnancy through 14 or 15 days during previous cycles during the same breeding season. These pregnancies were electively terminated by 15 days of gestation. Mares had no ultrasonographic evidence of uterine or ovarian pathology throughout this study.

Embryo recipient mares

Five pony mares, ages 2 - 7 years, and one light horse mare (age 11 years) were used as embryo recipients. Recipient mares were either young maiden mares or known fertile mares that were confirmed pregnant within the 12 months prior to this study. All pregnancies were terminated routinely during early gestation as part of another project and no intrauterine procedures were performed since that time. Mares had no ultrasonographic evidence of uterine or ovarian pathology throughout this study.

Breeding management and embryo recovery

Donor mares were presented daily to a stallion until behavioral estrus was identified. Beginning on the first day of estrus, the reproductive tract of each mare was examined between 7 and 9 AM daily by transrectal palpation and ultrasonography to monitor ovarian follicular development. Once the mare developed an ovarian follicle $\geq$ 35 mm in diameter, with associated uterine edema and a relaxed cervix, the mare received hCG (2500 IU, IV; Intervet, Millsboro, DE). The morning following hCG administration, mares were bred via artificial insemination of fresh extended semen collected from one of two resident stallions of proven fertility. Semen was extended 1:1 (v:v) with INRA 96 (IMV Technologies, Maple Grove, MN) and immediately used for breeding. Each mare was inseminated with a minimum of 1 x 10^9 progressively motile spermatozoa. Daily transrectal palpations and ultrasonographic examinations were continued until ovulation was detected. Most ovulations occurred within 24 hours after insemination and all occurred within 48 hours after insemination.

Day of ovulation was considered Day 0. Embryo recovery was performed as described,[11] with several modifications. Day 8 after ovulation embryos were recovered using a Y-junction tubing system (HarVet/Veterinary Concepts, Spring Valley, WI). The common port of the Y tubing was attached to a 34 french silicone catheter with a 60 cc balloon (HarVet/Veterinary Concepts). One end of the Y tubing was attached to a 65 µm embryo filter (MAI Genesis, Spring Valley, WI) and the other end was connected to a 2 liter bag of embryo flush medium (Vigro™ Complete Flush Media, Bioniche, Belleville, ON, Canada). The tubing was flushed and filled with medium prior to passage through the mare's cervix into the uterus. The mare's perineum was scrubbed three times with non-residue soap (Ivory®) and dried. Using sterile technique, the silicone catheter was passed through the cervix and the cuff inflated with 60 cc of air. One to 2 liters of embryo flush medium was used for each of three uterine lavages. For each lavage, transrectal ultrasonography was used to confirm that both uterine horns were fully distended with media prior to recovery of the fluid through the in-line embryo filter. Cloprostenol (250 µg, IM; Estrumate®, Merck Animal Health, Summitt, NJ) was administered to each donor mare following the embryo recovery attempt.

The embryo filter was searched under a dissecting microscope. Recovered embryos were transferred from the filter dish using a 0.5 cc straw and washed four times in embryo holding media (Vigro™ Holding Media, Bioniche).

Embryo collapse and vitrification

Embryos were placed in a small amount (~ 0.25 - 0.5 ml) of holding media in a standard petri dish under a dissecting microscope. The diameter of each embryo was measured before and after blastocoele puncture/collapse using the 'Measurement' function of the Standalone System (SAL) for an Olympus DP21 camera (Tokyo, Japan) attached to the dissecting microscope (Figure 1A-C). Briefly, the 'Scale Settings' on the SAL were set to coincide with the monitor adapter and microscope objective magnifications. The SAL measurements were obtained using a simplified function, based on the CCD pixels delineated by placement of an electronic cursor.

The embryo was held in place using very gentle aspiration applied to a tuberculin syringe attached to a 25 gauge needle (Sigma-Aldrich, Saint-Louis, MO). The blastocoele was punctured manually using a second 25 gauge needle attached to a tuberculin syringe. Because of the large size of the needle, the puncture was performed using a quick stabbing motion with an attempt to introduce only the tip of the beveled needle into the embryo. Rather than applying suction to the syringe used for puncture, blastocoele fluid was allowed to passively diffuse out of the hole created by the needle (Figure 1C).

Following blastocoele collapse, embryos were vitrified according to the manufacturer's recommendations, using a commercially available embryo vitrification kit (EquiPRO Vit-Kit™, MOFA Global, Verona, WI) adapted from a glycerol/ethylene glycol-based vitrification method previously described.[10] Embryos were stored in 0.25 cc straws in liquid nitrogen until warming and transfer the following breeding season.

Preparation of embryo recipients

Estrus was synchronized and/or hastened in recipient mares using either a compounded combination of 150 mg progesterone and 10 mg estradiol IM once daily for 10 days (Hagyard Pharmacy, Lexington, KY) and 250 µg of cloprostenol IM on day 10 (n = 2 pony mares) or a single injection of 250 µg of cloprostenol IM (n = 3 pony mares and 1 light horse mare). Once uterine edema was identified in association with a $\geq$ 30 mm follicle, the reproductive tract of each mare was examined daily or every other day by transrectal palpation and ultrasonography to monitor ovarian follicular development. Once the mare developed a follicle $\geq$ 35 mm, with associated uterine edema and a relaxed cervix, the mare received hCG (2500 IU, IV). Transrectal palpation and ultrasonographic examinations were continued, daily or every other day, until ovulation was detected.

Embryo warming and transfer

Five or 6 days after ovulation was documented in the recipient mare, a single vitrified embryo was warmed and transferred transcervically. Embryos were warmed in the closed 0.25 cc straw in a 37ºC water bath for 60 seconds. After warming, the straw was flicked to mix the contents and contents expelled into an empty well of a 4 well plate at 37ºC. Using a 0.5 cc straw attached to a 1 ml syringe, the embryo was then transferred through a series of embryo holding media and sucrose solutions, as follows: 0.3 M sucrose for 1 minute, 0.15 M sucrose for 5 minutes, and holding media alone for 5 minutes (Figure 2A-D; modified from the "EG/s" method described[13]).

Warmed embryos in holding medium were then aspirated into a 0.5 cc straw. The straw containing the embryo was loaded into an embryo transfer gun (Bioniche) and the gun was covered with a sterile sheath with side delivery ports for 0.5 cc straws (Bioniche). Transcervical transfer was performed routinely as previously described.[11] After passage of the transfer gun through the cervix, placement of the tip of the gun at the base of a uterine horn was confirmed by palpation per rectum prior to deposition of the embryo into the uterus. The tip of the sheath was examined under a dissecting microscope following transfer to confirm that the embryo had been expelled. Unlike advanced embryo manipulations, all embryo transfers were performed by one highly experienced clinician.

Pregnancy examination

Pregnancy diagnosis was performed via transrectal ultrasonographic examination of the uterus at least twice between 7 and 30 days after transfer.

Results

Embryo recovery, collapse, and vitrification

Six donor mares were bred via artificial insemination over 17 estrous cycles. Fifteen embryos were recovered (88% per cycle embryo recovery rate). Only embryos measuring $\geq$ 290 µm and classified as Grade I were used for manual blastocoele collapse and subsequent vitrification (n = 12). The remaining three embryos were either classified as Grades II or III. Embryos used for blastocoele collapse and vitrification ranged in size from 290 - 725 µm.

All 12 embryos were readily held in place and punctured manually in < 5 minutes. Once the needle was removed the resulting hole in the trophoblast was large enough to allow > 50% of the blastocoele fluid to diffuse passively out of the embryo over several minutes, without applying suction to the syringe (Figure 1). Vitrification was done by one of three veterinarians in residency training. These individuals had prior experience with fresh embryo transfer, but had no experience with embryo collapse or vitrification.

Embryo warming and transfer

Two straws containing vitrified embryos were broken when retrieved from the liquid nitrogen storage tank. An additional 2 straws fractured during warming in the water bath. Embryos were not recovered from any of these 4 straws. After warming, 1 embryo was lost during transfer among sucrose solutions. Finally, after passage through the sucrose solutions, 1 embryo had the appearance of an empty embryonic capsule containing no trophoblast or inner cell mass. This structure was not transferred. Problems associated with these 6 straws/embryos resulted in a 50% embryo loss rate prior to transfer. Six embryos were successfully passed through the sucrose gradient and transferred into holding media prior to routine transcervical transfer. None of the transferred embryos resulted in pregnancies at 7 - 30 days after transfer. Data are summarized in Table.

Discussion

The objective was to develop a simplified method for vitrification and warming of expanded equine blastocysts that could be used by practitioners with no prior experience in embryo collapse, vitrification or warming and that would result in pregnancies following embryo transfer. The method chosen was designed to mimic a method described by several field practioners. The technique was simple and did not require expensive laboratory equipment; however, it was associated with a high percentage of embryo loss during storage, warming and processing (50% embryo loss) and resulted in no pregnancies following embryo transfer.

Others have described adequate PRs following embryo collapse, vitrification and warming.[13,15,16] However, in an attempt to adapt this process for more general use, our chosen methodology differed in one or more ways from each of these more successful methods. Differences included the experience of the individuals manipulating embryos and the methods used for embryo puncture and collapse, vitrification and embryo warming and rehydration.

To mimic the situation most often encountered in practice, all advanced embryo manipulation procedures were performed by individuals with experience manipulating fresh embryos, but with no experience performing embryo collapse, vitrification, warming or rehydration. After reviewing the vitrification protocol, all were able to complete the collapse and vitrification process readily on the first attempt without assistance, attesting to the ease of this approach. However, 4 straws were broken either upon removal from the liquid nitrogen storage tank or during warming, suggesting that these straws may not have been properly loaded or sealed. Additionally, moving the collapsed/vitrified/warmed embryos through the sucrose gradient proved very challenging for individuals unfamiliar with manipulating embryos in hyperosmotic solutions. Unlike commercial embryo flush and holding media in which embryos readily sink to the floor of the petri dish, hyperosmotic rehydration gradients cause the embryo to float in suspension for a time until reaching equilibrium and subsequently falling to the floor of the dish. Because of these differences, and in spite of the relative simplicity of this technique, operator inexperience still likely contributed to the high percentage of vitrification and embryo loss after vitrification. We inferred that veterinarians experienced only with commercial, fresh embryo transfer may initially find even this simplified technique difficult to execute.

To insure that the embryo transfer technique was controlled and would not contribute to a reduction in subsequent pregnancy rates, all embryo transfers were performed by a clinician highly experienced with commercial embryo transfer and with a history or achieving a fresh embryo transfer PR > 85%. Similarly, donor/recipient asynchrony was very good and therefore also was unlikely to contribute to a reduction in pregnancy rates. By examining recipient mares daily or every other day, we were able to ensure that embryo transfer was performed on days 5 or 6 after recipient mare ovulation. Pregnancy rates are optimal and not significantly different when day 8 embryos are transferred on either days 5 or 6 after the recipient mare's ovulation. Similarly, pregnancy rates are optimal and not significantly different when day 8 embryos are transferred to recipients ovulating 2 or 3 days after the donor.[1,17] Since embryos were obtained and vitrified on day 8 after donor mare ovulation, our recipient mares ovulated the equivalent of 2 or 3 days after donors.

To eliminate the need for micromanipulation equipment, we used small-gauge hypodermic needles attached to tuberculin syringes both to hold the embryo in place and to manually puncture and collapse the blastocyst. Even though the holding needle was not purposefully blunted, we observed no apparent damage to the embryo when gentle suction was applied to stabilize the embryo on the tip of the holding needle. However, slight movement of the embryo was noted during subsequent blastocoele puncture, suggesting that this approach did not immobilize the embryo as effectively as a micro holding pipette. After puncture of the embryonic capsule and trophoblast with the hypodermic needle, the resulting hole was large enough to allow for passive diffusion of > 50% of the blastocoele fluid, without suction. However, in previous studies on large equine embryos in which the blastocoele was collapsed more extensively using micromanipulation, PR > 83% were reported following vitrification, rehydration and subsequent embryo transfer.[11] Similarly, removal of as much as 87 - 100% of the blastocoele fluid prior to vitrification of human embryos has resulted in the highest embryo survival rates.[18–20] It is therefore possible that our passive diffusion method contributed to pregnancy failure by inadequately collapsing the blastocoele.

Another factor that potentially contributed to the failure of our approach was the size of the needle used for blastocyst puncture. The outer diameter of a 25 gauge needle (~ 510 µm) is > 25 times the size of the outer diameter of most glass microinjection needles (~ 20 µm , Figure 3) and the use of even the tip of the bevel resulted in the creation of a large hole in the embryonic capsule and trophoblast (Figure 1). In this regard, one embryo appeared as an empty embryonic capsule after warming, suggesting that the trophoblast and inner cell mass may have been lost through the punctured capsule. The embryonic capsule protects the embryo during the motility stage of intrauterine development and damage to the capsule will reduce embryo survival following transfer.[21] Although more expensive and more fragile than a standard injection needle, glass microinjection needles are available commercially and could be used to manually puncture an equine blastocyst without the need for a micromanipulator. The limitations imposed by the purchase and storage of microinjection needles may be outweighed by their smaller size and the potential reduction in damage to the embryo during puncture. However, the size of the 25 gauge needle is not likely to be the sole cause for pregnancy failure, since a 46.7% PR (7 of 15) was reported following transcervical transfer of vitrified embryos manually punctured with a 25 gauge needle and vitrified using a low-volume, open vitrification system.[16]

The method of vitrification/warming likely has an important role in embryo survival. We used a vitrification/warming system modified from a previously described ethylene glycol/sucrose (EG/s) method.[10,13] By combining micromanipulator-assisted blastocoele collapse with the EG/s vitrification/warming solutions and a low-volume, open vitrification/warming system performed in microloader tips, PRs increased to 86% after embryo transfer.[13] In an attempt to simplify vitrification, we substituted a closed system in 0.25 ml straws. Embryos then were warmed in the closed straws before being expelled to permit stepwise rehydration of the collapsed blastocoele in an open system. Although similar closed vitrification/warming systems have resulted in acceptable PRs when used on small, intact blastocysts or morulae, this study and others have reported no pregnancies using expanded equine blastocysts (> 400 µm) with or without collapse of the blastocoele.[10,12-14,16] All reports to date describing good PRs following collapse and vitrification of expanded equine blastocysts used an open vitrification/warming system, combined with a fine-diameter microloader pipette tip.[13,15,16] Small volume (< 1 µl), open systems facilitate faster and more even cooling and warming rates and have been speculated to help minimize ice crystal formation.[5,14,22] Given the success rates reported to date, open vitrification systems combined with microloader tips and stepwise rehydration of the collapsed embryo appear to be critical to embryo viability after warming.

Conclusions

Our attempt to simplify the methods for collapse and vitrification of expanded equine blastocysts was unsuccessful in that it resulted in no pregnancies following embryo transfer. Additional work needs to be done to develop simplified methods of collapse that could be combined with straightforward methods of vitrification and warming to permit preservation of expanded equine blastocysts in a clinical setting. It is

possible that both the use of the 25 gauge needle for blastocoele collapse and the use of a closed, relatively large-volume system for vitrification/warming contributed to the absence of pregnancies. Based on our results, the technique as we described, cannot be recommended for clinical use.

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Conflict of Interest

Authors have no conflicts of interest to declare.

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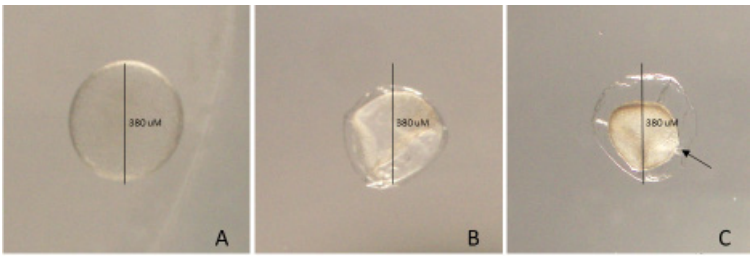

Figure 1. Appearance of day 8, Grade I blastocyst prior to puncture (A), 1 minute after puncture (B) and 5 minutes after puncture (C). Note collapse of the blastocoele within the embryonic capsule in Panels B and C. The hole in the trophoblast was most apparent in Panel C (marked by an arrow). Measurements were obtained using the 'Measurement' function of the Standalone System (SAL) for an Olympus DP21 camera.

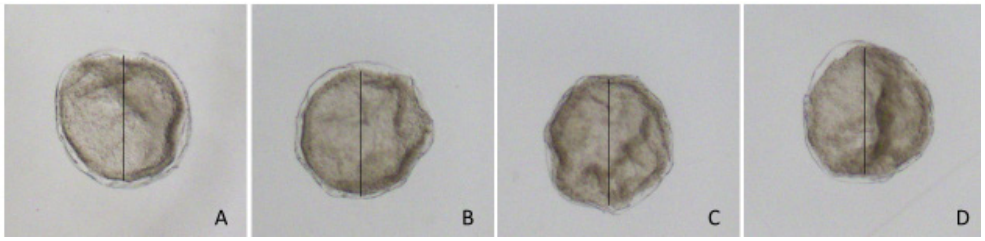

Figure 2. Appearance of a collapsed embryo immediately after warming and after mixing of the contents of the warmed straw (A), after transfer into the 0.3 M sucrose solution (B), after transfer into the 0.15 M sucrose solution (C), and after transfer into holding media prior to transfer (D). The bar in all four panels is 600 µm. Prior to collapse and vitrification, this embryo was measured as a 725 µm Grade I blastocyst.

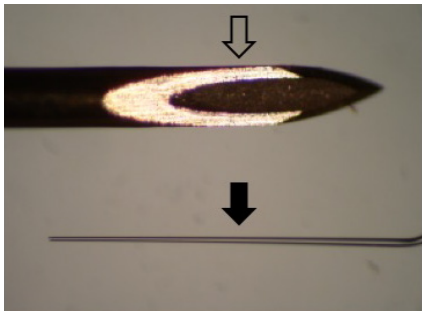

Figure 3. Comparison of the outer diameter of a 25 gauge hypodermic needle (open arrow, top) and a 20 µm microinjection needle (closed arrow, bottom).

Table. Equine embryo production, recovery and transfer.

Estrous cycles	Embryos recovered	Embryos collapsed and vitrified	Embryos transferred	Pregnancies
17	15 (88%)	12 (71%)	6 (35%)	0 (0%)