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Embryo transfer in Indian standardbred mares

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ABSTRACT

This study was conducted for 3 years. A total of 81 standardbred mares were used as embryo donor. Single embryo collection regime was attempted in 117 times using 3 liters of Gold Flush, Line, Euro Flush, and 95 embryos were recovered with recovery rate of 95/117=81.19% (embryo /flush). 48 embryos were transferred into synchronized recipients (+1 to –2 days) and the overall pregnancy rate at day 24 achieved was 50% (24/48). The birth of foal on 6 February 2006 through embryo transfer was reported first time in South Asia.

Key words: Equine embryo transfer, Standardbred mares

Equine embryo transfer (ET) is becoming popular for obtaining foals. There are several reasons for the use of this procedure as multiple foals can be produced from one mare (possibly genetically superior) in a breeding season; to produce foals from mares that cannot take time off from racing or showing; to produce foals from sub-fertile mares that are unable to successfully carry a foal to term Squires (1993); to get foals from young fillies that can produce viable embryos, but are not able to carry them; foal from older mares no longer capable of carrying a foal; to produce offspring from endangered equids such as Przewalski's horse (Summers *et al.* 1987), zebras and endangered Poitou donkey (Mckinnon 2005), and to cut down on breeding injuries and the spread of venereal diseases in the horses (Mckinnon and Squires 2007).

The development of equine ET has lagged behind due to the restrictions imposed by most of the major breed registries. In 2002 the American Quarter horse association acting on legal advice, withdrew the rule governing the number of embryos transferred per mare per year and retrospectively allowed registration of multiple ET derived foals from the beginning of the introduction of ET to AQHA legislation (Mckkinon and Squires 2007). The study was carried out with objective to establish embryo transfer in Indian standard mares to keep pace with developing countries in equine reproduction research.

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MATERIALS AND METHODS

Selection of donor and recipient mares: Standarbred horse mares (n=81 in 3 years study) between 4 and 14 years of age were selected on the basis of their sound breeding records and transrectal ultrasonography of genital tract. Mares were in good body condition and kept under ideal managemental conditions at Babugarh, Army Stud Farm, Ghaziabad,Uttar Pradesh, in northern India.

The time of ovulation of these selected mares was ascertained by frequently examining the Graffian follicles by trans – rectal ultrasonography at 5 MHz linear probe. The mares were inseminated artificially with extended fresh semen of known fertile stallion assuring a minimum of 500 million live spermatozoa.

Recipient mares were selected on the basis of good breeding history and ultrasonographic examination of genitalia. Synchronization of estrous of recipient and donor mares was done by intramuscular injection of $PGF_2\alpha$ 1 ml. Estrus was detected with the help of teasing pony in the early morning and evening followed by the palpation of genital organs rectally. The donor and recipients mares were examined daily as per the synchronization schedule and the day of rupture of follicle was counted as day 0.The embryos were flushed non- surgically on day **7** of ovulation.

Embryo collection via non-surgical methods: At the time of collection, donor mares were scrubbed and washed in the perineum with antiseptic solution after the back racking of rectum. 10–20 ml Xylazine Hydrocloride was injected intramuscularly. The mares were flushed with 3 litres of equine embryo flushing medium, using 3 consecutive uterine flushing with 800 ml, 1000 ml and 1200 ml of fluid using 2 way 20 Fr/30cc 26" silicone Foley's catheters and embryos

were recovered on day 7 of ovulation. Balloon of Foley's catheters was fixed on cranial side of cervix. The embryos thus collected were washed at least 10 times in holding medium (DPBS plus 0.4% BSA fraction-V) filtered through 0.22µm filter. The diameter of the embryos were measured with the help of micrometer and evaluated morphologically at room temperature under a stereoscopic microscope as described by McKinnon and Squires (1988a).

Grading of embryo was done by using stereoscopic microscope as described by McKinnon and Squires (1988a).

Grade 1: Excellent Spherical Uniform cell size, colour and texture

Grade 2: Good Minor Imperfections Few extruded blastomeres, irregular shape, trophoblastic separation

Grade 3: Fair Obvious problems Extruded blastomeres, degenerate cells, collapsed blastocele

Grade 4: Poor Severe problems, Oblong and irregular Many extruded blastomeres, collapsed blastocele degenerate cells.

Embryo transfer in recipient mares: The embryos were transferred by non-surgical technique though the cervix in synchronized recipients (+1 to –2 days) by the same operator. The embryos were drawn into a 0.25 ml straw and placed into a stainless steel cassou-type gun covered with a sterile plastic sheath incorporated with a metal plug that fitted over the straw and directed the embryo out of the device through either side ports Squires and Seidel (1995). This assembly was again covered with a sanitary plastic sheath to prevent contamination. The embryos were placed between columns of air and medium to minimize movement within the straw as well as to ensure expulsion of the embryos. The tail of the recipient mare was wrapped, faecal contents were removed and the genitalia cleaned. During transfer the technician wore a sterile plastic sleeve. The transfer gun was gently placed into cervix and sanitary sterile sheath was penetrated and embryo deposited in the uterine body. At the time of embryo transfer all the recipients were given 500 mg of hydroxy progesterone caproate and 900 mg of phenylbutazone intramuscularly. Phenylbutazone was given to prevent the release of prostaglandin during the cervical manipulation during transfer of the embryo.

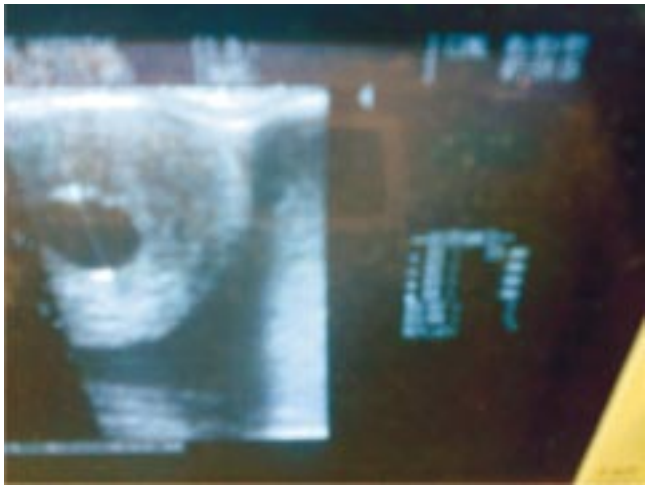

Fig. 1. 16-day-old pregnancy in a recipient mare.

Confirmation of pregnancy: Diagnosis for pregnancy was done on day 16 and 20 with the help of ultrasound through rectal probe of 5.0 MHz (Fig. 1). Mares found pregnant were confirmed again on day 35 by rectal palpation as well as by examining with the help of an ultrasound scanner. The proportional data were analyzed by Chi-Square test and with an analysis of variance (ANOVA) as described by Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

In the three-year study total 81 mares were used as embryo donor. Single embryo collection regime was attempted 117 times and 95 embryos were recovered with recovery rate of 95/117=81.19% (embryo /flush) (Table 1).

Grading of embryos: Recovery rate of grade 1 embryos was 63.13% (60/95), grade 2 embryo 27.35% (26/95), grade 3 embryo were 4.21% (04/95) and grade 4 embryo were 5.26% (05/95) (Table 1). Recovery of grade 1 embryos was 60 (63.13%) which was significantly higher ($P<0.05$) than any other grades and resulted in 56.25% (18/32) pregnancy rate at day 24. The recovery of grade 2 embryos was significantly lower than grade 1, but higher than grade 3 and 4. 16 embryos in grade 2 were transferred and 06 pregnancies were obtained on day 24. The grade 3 and 4 embryos recovered were not transferred. In the present study, 48 embryos were transferred into synchronized recipients (+1 to –2 days) and the overall pregnancy rate at day 24 achieved was 50% (24/48).

Stage of embryos: Out of 95 embryos recovered, the morula stage was found in 50.52% (48/95), early blastocysts 27.36% (26/95), expanded blastocysts 15.78% (15/95), hatched blastocysts 6.31% (06/95) (Fig. 2). In early blastocysts the pregnancy rate at day 24 was significantly higher 12/17 (70.5%) ($P<0.05$) than the pregnancy rates in morulae (46.6%), expanded blastocysts (36.3%) and hatched blastocysts (20%).

Size of embryos: Overall size of embryos fluctuated between 150µm to 1200µm. Recovery rate of embryos of size 100–299 µm was 71.57% (68/95), for size 300–599 µm, it was 23.15% (22/95), for size 600–999 µm it was 3.15% (03/95) and for 1000 µm it was 2.1% % (02/95). The recovery of the embryo of 100µm to 299µm size was significantly higher 68/95 (71.57%) than 300 µm size 27/95 (28.42%). Pregnancy rate at day 24 was significantly higher 17/31 (54.8%) ($P<0.05$) for smaller size embryos than 300 µm embryos 7/17 (41.17%) (Table 1).

Out of total 95 embryos recovered only 48 embryos were

Table 1. Analysis of embryos according to morphological grade, development stage and size of embryos

Grade/stage/size	Embryos collected	Fresh embryos transferred	Pregnant (day 24)	Embryos frozen	Embryos discarded
Grade					
Grade 1	60 (63.13%)	32	18(56.25%)	28	-
Grade 2	26 (27.35%)	16	06(37.5%)	10	-
Grade 3	04 (4.21%)	-	-	-	04
Grade 4	05 (5.26%)	-	-	-	05
Stage					
Morula	48 (50.52%)	15	07(46.6%)	26	07
Early blastocyst	26(27.36%)	17	12(70.5%)	08	01
Expanded blastocyst	15 (15.78%)	11	04(36.3%)	04	-
Hatched blastocyst	6 (6.31%)	05	01(20%)	-	01
Size					
100–299 µm	68 (71.57%)	31	17(54.8%)	30	07
300–599 µm	22 (23.15%)	14	05(35.7%)	07	01
600–999 µm	3 (3.15%)	01	01(100%)	01	01
≥ 1000 µm	2(2.1%)	02	01(50%)	00	

P, <0.05.

transferred fresh and rest of embryos were frozen as apart of other study or discarded being of lower quality. Out of 48 recipients, 24 mares were found pregnant on day 24 (50%), out of them 14 (29.16%) carried pregnancy to term and healthy foals were born. Three mares aborted after 3 months of pregnancy and 07 mares slipped foal before 95 days. All foal were healthy except one suffered from unilateral congenital anopthalmia. Average gestation length was 331 days. Out of total 14 foals born, ratio of Colt and Filly was 8: 6. The average birth weight of foals was 39.64 kg. Average height was 91.28 cm, average girth was 74.78 cm, and average shank was 10.14 cm.

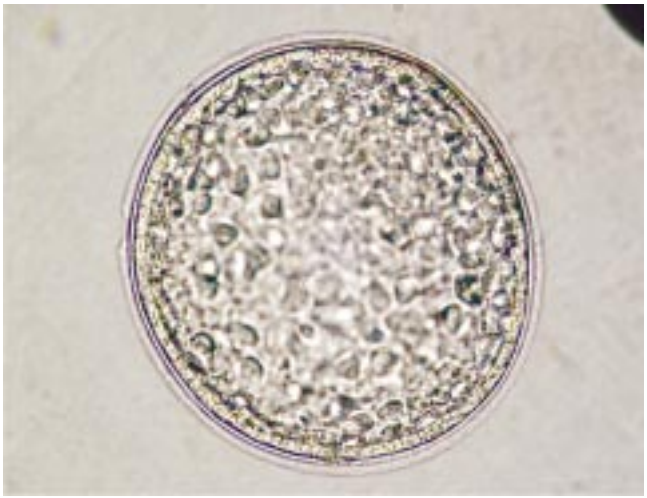

Fig. 2. 450 µm expanded blastocysts grade 1.

In a commercial embryo transfer programme, it is important to develop techniques that optimizes embryo recovery rate and decrease the time and cost involved in collection and transfer of equine embryos Fleury and Alvarenga (1999). Embryos are usually collected on day 6, 7 or 8 (day 0 is day of ovulation) from naturally single or occasionally multiple ovulations. Methods of recovery have not varied much from the original description of Imel *et al.* (1981). Success of commercial equine embryo transfer depends on the efficacy and economy of collection of embryo from donor animals. Mean embryo recovery per cycle from single ovulating mares in equine embryo transfer programme ranges from 50 to 80% (Squires 1993, Peres *et al.* 1992, Jacob *et al.* 2002, Taveiros *et al.* 2002, Squires *et al.* 2003). The embryo recovery rate (embryo/flush) in the present study was 95/117 (81.19%). Lascombes and Pashen (2000) reported similar embryo recovery rate for a commercial embryo transfer programme.

In early publications on ET, researchers advocated the use of surgical transfer of equine embryos as various studies on non-surgical transfer of equine embryos have been attempted by others with extremely variable results (12.5%–71% pregnancy rates: Vogelsang *et al.* 1979, Oguri and Tsutsumi 1980,Castleberry *et al.* 1980 and Douglas 1982). Currently they restrict the use of assisted reproductive technologies (such as ICSI, GIFT or oocyte transfer) to surgical transfer because pregnancy rates have been greater than 75% per embryo transferred at day 15 for more than 8 years. Many variations of non-surgical ET have been reported by Silva *et al.* (2004) and Wilsher and Allen (2004). However careful management of delicate tissues in standard non-surgical ET can only result in consistent pregnancy rates McKinnon and Squires (2007).

Pregnancy rates as reported in literature ranges from 49.5% to 74.55% of embryos transferred (Jasko 2002, Taveiros *et al.* 2002, Fleury and Alvarengra 1999, Peres *et al.* 1992). In the present study, the pregnancy rate achieved at day 24 was 50% (24/48). Squires *et al.* (1985) reported higher

pregnancy rates when embryos were transferred by guarded pipette method or Cassou gun method as done in present study and also suggested that bigger embryos having their increased fluid volume to surface ratio has lower pregnancy rate. Vogelsang *et al.* (1985) reported higher pregnancy rates from embryos of day 6 or 7 as compared to embryos of the age of day 8, 9 and 10. Rocha *et al.* (2004) transferred non-surgically 152 embryos to cycling mares 4 to 8 days after ovulation and got 75% pregnancy rate on day 12.

Morphologic evaluation has been described as an objective assessment of embryo viability and probability of establishing pregnancy after transfer Carney *et al.* (1991), Mc Kinnon and Squires (1988a) and Vanderwall (1996). Morphological grade of the embryo is the most important factor to establish a pregnancy and minor flaws in embryo morphology results in reduced viability Carnevale *et al.* (2000). In an embryo transfer programme, the embryo morphology cannot be improved; however, the exposure of the embryo to toxins, improper temperature or medium could adversely affect morphology, grade and viability. Embryo morphology is the predictive of success of embryo transfer programme.

Grade of an equine embryo before transfer dramatically affects pregnancy rates as reported in cattle Elsden and Seidel (1990). The recovery of grade 1 embryos in the present study was 63.13% (60/95) and pregnancy rate achieved in grade 1 embryos was 56.25% . The similar finding was reported by Mc Kinnon and Squires (1988b), Mc Kinnon *et al.* (1988), Fleury *et al.* (1989), Squires *et al.* (1992), Fleury and Alvarenga (1999), Jacob *et al.* (2002).Huhtinen *et al.* (1997) and Carney *et al.* (1991) also reported higher pregnancy rates for Grades 1 and 2 embryos as compared to Grades 3 and 4 embryos. The present study is in agreement with most of other studies. Clark *et al.* (1987) reported 13 of 26 successful transfers with embryos of less than grade 2 compared with 1 of 6 pregnancies from transfer of grade 3 or greater. The pregnancy rates on day 50 after surgical transfer for embryos given a quality score of 1 or 2 (69.0%) were better ($P<0.05$) than those for embryos graded ≥ 3 (22.18%) (McKinnon *et. al.* (1988). Carney *et al.* (1991) also reported higher pregnancy rates for Grades 1 and 2 embryos compared with Grade 3 and 4 embryos.

In the present study, the pregnancy rates of morulae and early blastocysts were 46.6% and 70.5% respectively, however, in case of expanded blastocysts, it was only 36.3%. In contrast to present study in a report on factors effecting pregnancy, it was higher for early blastocysts, blastocysts and expanded blastocysts (62.2, 72.9 and 68.7% respectively) as compared to morulae (47.1%) (Carnevale *et al.* 2000). Size and age of embryos have been reported to be highly correlated Luliano *et al.* (1985).

Fewer pregnancies were obtained after non-surgical transfer of embryos of size 1250 to 2830 µm diameter compared with those of embryos of 400 to 1240 µm. The same authors reported that size of embryo (360 to 1760µm) did not influence pregnancy rate after surgical transfer unless the embryo was greater than 2500 µm. Fleury *et al.* (1989) reported similar pregnancy rates for embryos of 200 to 500 µm, 501 to 1000 µm,1000 to 1700 µm and 1701 to 4200 µm in diameter. In the present study, pregnancy rate of smaller size (100–299 µm) embryos was significantly higher 54.8% as compared to larger size (300–599µm) embryos (35.7%). It may be due to the factor that large size embryos may be less resistant to temperature stress and osmolarity changes than smaller embryos (Luliano 1983). The larger and aged embryos with their increased fluid – volume to surface ratio may not withstand the shock of recovery and transfer to the recipients as compared to smaller embryos (Mc Kinnon and Squires 2007).

The birth of foal through embryo transfer was reported in South Asia. The embryo recovery rate was encouraging. There is further need to improve this technique as per Indian conditions so that horse owners at village level can be benefited.

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