

Non-surgical transfer of 4',6'-diamidino-2-phenylindole-stained equine embryos

Mirja Huhtinen

*Agricultural Research Centre of Finland, Equine Research, FIN-32100 Ypäjä, Finland,
e-mail: mirja.huhtinen@mtt.fi*

Peter Bredbacka

Agricultural Research Centre of Finland, Institute of Animal Production, FIN-31600 Jokioinen, Finland

4',6'-diamidino-2-phenylindole (DAPI) is a fluorescent dye that binds only to the DNA of dead blastomeres of an embryo. The use of DAPI for assessing viabilities of equine Day 6 to 7 embryos was studied. The pregnancy and normal development of the embryo until 20 days after transfer (day of pregnancy termination) was used as an indicator of embryonic viability. Eleven embryos were stained with DAPI at room temperature for 15 min. They were then exposed to UV light to visualize staining, and cultured for 2 h before non-surgical transfer to recipient mares. Eight control embryos were cultured for 2 h before transfer to recipient mares. The recipient mares were scanned for pregnancies every other day starting 6 days after transfer. Twenty days after the transfer the pregnant recipient mares received luprostitol to induce abortion of the foetus. The pregnancy rate 6 days after transfer was 82% in the treatment group, and 75% in the control group ($P>0.05$). One DAPI-stained embryo resorbed and was no longer visible 12 days after transfer. However, this particular recipient mare had a uterine inflammation as shown by examination in the oestrus following embryo transfer. Although the number of embryos is insufficient to demonstrate lack of treatment effect, the high pregnancy rates following DAPI staining should encourage further studies on this subject.

Key words: embryo quality, DAPI, vital staining

Introduction

Morphological assessment of an embryo using a high-quality microscope is usually sufficient for deciding which embryos are worth transferring or freezing. There are situations, however, when

the difference in quality cannot be distinguished by morphological criteria only. A quick and easy viability test would be desirable in practical situations and also when new techniques in embryo technology, e.g. freezing, are developed. Metabolic tests measuring glucose, glutamine or oxygen consumption have been developed for

horse and cattle embryos (Rieger et al. 1987, Rieger and Guay 1988, Brück and Hyland 1991, Overstrom et al. 1992). They seem to work under laboratory conditions, but are time consuming and require delicate analysis techniques. Less time and equipment are needed to evaluate embryo quality using vital stains.

Several workers report using a vital stain to test the viability of an embryo. Examples of non-fluorescent dyes are neutral red (Kardymowicz 1972) and trypan blue (Hutz et al. 1985, Majumdar 1990). Some fluorescent dyes, e.g. fluorescein diacetate (FDA), stain live cells (Schilling and Doepke 1978, Schilling et al. 1979a, Mohr and Trounson 1980, Schilling et al. 1982, Pruitt et al. 1988); others, e.g. 4',6'-diamidino-2-phenylindole (DAPI), dead cells of the embryo (Schilling et al. 1979b, Low et al. 1986). Rhodamine 123, a mitochondrion-specific fluorescent dye, may also be useful for assessing the metabolic integrity of mammalian embryos. This stain has, however, been shown to have cytotoxic effects if exposed to UV light for more than 5 min (Petters and Lucy 1987).

Only FDA (Pruitt et al. 1988, 1991) and DAPI (Huhtinen et al. 1995) have been used to assess the viability of equine embryos with fluorescent stains. A high pregnancy rate was reported (Huhtinen et al. 1995) following transfer of whole equine embryos after treatment with cytochalasin B and nocodazole, and DAPI staining. Transrectal ultrasonographies between Days 12 and 25 revealed that seven of eight whole embryos produced embryonic vesicles, but two of these exhibited growth retardation, and were expelled from the uterus as the recipient mares returned to oestrus. The retarded growth exhibited by two out of seven embryos in that group suggested possible detrimental effects due to the compounds used or exposure to UV light. As DAPI staining might be useful for diagnosing the viabilities of embryos after treatments such as freezing and thawing, the toxicity of that staining alone would have to be examined. The aim of our study was to establish whether DAPI could be used for staining fresh Day 6 to 7 equine embryos before transfer, without compromising further development of the embryo.

Material and methods

The study was carried out in March and April 1994 with 35 Finnhorse and 23 Warmblood mares 3 to 18 years old (mean age 8.7 years). One to three cycles per mare were used in the experiment. Each mare served as donor or recipient or both. Two Finnhorse stallions of known fertility were used for inseminations.

Oestrous cycles were synchronized by injecting 7.5 mg of luproliol i.m. (Prosolvin, Intervet International B.V., Boxmeer, Holland) in dioestrus. Ovulations were confirmed by rectal palpation and daily transrectal ultrasonography. Freshly collected semen was diluted with Kenney's skim milk/glucose extender. Inseminations were undertaken every other day before ovulation, using more than 500 million progressively motile spermatozoa per dose. The ovulations of recipients were induced, when necessary, by administration of 2500 IU hCG (Chorio-Gonadon, Orion-Farmos, Turku, Finland) i.v. in oestrus.

Embryos were recovered 6 days after detection of ovulation by flushing the uterus three times with 1 L of PBS supplemented with BSA (2 g/L) and kanamycin (25 mg/L). Flushing medium recovered from the mare was passed through an embryo filter (Emcon - Immuno Systems, Spring Valley, WI, USA), and the fluid in the filter cup was examined for the presence of embryos using a stereomicroscope. Embryos were washed and transferred at room temperature to Hepes-buffered (25 mM) Ham's F10 (Gibco BRL, Life Technologies, Paisley, Scotland) supplemented with 10% foetal calf serum (Gibco BRL), 50 IU/ml of penicillin, and 50 µg/ml of streptomycin. Embryos were classified according to McKinnon and Squires (1988). Diameters were measured using an eyepiece graticule. All the embryos were photographed on a black-and-white film and on a videotape before staining and/or culture.

Nineteen randomly selected embryos were used in this experiment; other embryos were submitted to another experiment.

Table 1. Description of DAPI-stained embryos transferred in this study.

number	diameter, µm	stage	quality grade	UV time, sec	number of stained cell	pregnancy
1	160	EB	1	14	1	–
2	160	EB	1	12	2	+/-
3	170	EB	1	14	2	+
4	160	EB	2	12	3	+
5	200	B	2	10	1	–
6	160	EB	1	11	0	+
7	170	EB	1	11	2	+
8	170	EB	2	13	5	+
9	170	EB	2	12	3	+
10	220	B	1	10	0	+
11	180	B	1	13	0	+

Stage: EB = early blastocyst, B = blastocyst.

Quality grade: 1 = excellent, 2 = good.

Pregnancy: + = pregnant, – = nonpregnant, +/- = early embryonic death.

Treatment group: Embryos (n=11) were placed in Hepes-buffered Ham's F10 solution containing 1 µg/ml DAPI (Sigma Chemical Co., St. Louis, MO), and kept at room temperature for 15 min. The staining was visualized using an inverted microscope (Olympus, IMT-2) with a dichroic mirror unit (IMT2-DMU: high pressure mercury burner, wavelength 334–365 nm, fluorescent radiation 420 nm and up) and a 20 x objective. Ultraviolet irradiation (10–14 s) of DAPI-stained embryos was recorded on a videotape. Fluorescing cells were considered dead, and were counted from the videotape images. After staining, the embryos were washed three times and placed in bicarbonate-buffered Ham's F10 (Flow Laboratories, Irvine, Scotland) supplemented with 10% foetal calf serum (Gibco BRL), 2 mM of L-glutamine (Gibco BRL), 50 IU/ml of penicillin, and 50 µg/ml of streptomycin. They were cultured in 5% CO₂ in air at + 37°C for 2 h before being transferred into uteri of recipient mares that had ovulated 0 to 2 days later than the donor. The embryos were loaded into 0.25-ml plastic straws and transferred via the cervix using a Cassou insemination instrument protected with an aseptic plastic shield.

Control group: Embryos (n=8) were washed three times and placed in bicarbonate-buffered

Ham's F10 (Flow Laboratories) supplemented with 10% foetal calf serum (Gibco BRL), 2 mM of L-glutamine (Gibco BRL), 50 IU/ml of penicillin, and 50 µg/ml of streptomycin. They were cultured in 5% CO₂ in air at + 37°C for 2 h before being transferred nonsurgically into the uteri of recipient mares that had ovulated 0 to 2 days later than the donor.

Uteri of the recipient mares were scanned every other day for embryonic vesicles starting 6 days after transfer. Twenty days after transfer the pregnant mares received 7.5 mg luproliol (Prosolvin) i.m. to abort the foetuses.

One-way analysis of variance was used for statistical analyses to compare sizes or morphological grades of embryos, or times from embryo recovery to transfer between the two groups. χ^2 -test was used to compare pregnancy rates after transfer.

Results

The embryo recovery rate was 64% (35/55). Fifty-five flushes yielded 37 embryos (including two sets of twins). The embryos used in this ex-

periment were randomly assigned to treatment and control groups. The mean diameter of embryos was 175 μm (range 160 to 220 μm) in the treatment group and 213 μm (range 160 to 360 μm) in the control group. The diameters of embryos in the two groups were not significantly different ($P>0.05$). All embryos were classified as excellent or good. The quality grade of embryos did not have a significant effect on the survival of the embryo after transfer, nor was it statistically different in the two groups ($P>0.05$). A more detailed description of DAPI-stained embryos is presented in Table 1.

Ultraviolet exposure time was 10–14 s (mean 12 s). The average number of dead cells was 1.7 (range 0–5). The number of dead cells did not have a significant effect on survival after transfer. The time from embryo recovery to transfer was 3.0 h ($\text{SD}\pm 0.4$ h) in the treatment group and 2.7 h ($\text{SD}\pm 0.3$ h) in the control group. This difference was not statistically significant ($P>0.05$).

Transrectal ultrasonography 6–20 days after embryo transfer revealed that 9 of 11 DAPI-stained embryos produced embryonic vesicles and the heartbeat was observed in eight of them before the induction of abortion. The embryo that did not develop further was no longer in the uterus by the time of the fourth examination (12 days after transfer). A cytological smear from uterine swab taken from this particular recipient 19 days after transfer revealed uterine inflammation. In the control group, six of eight embryos survived transfer and in all of them the heartbeat was observed before abortion induction 20 days after transfer. There was no difference in pregnancy rates 6 or 20 days after transfer between treatment and control groups ($P>0.05$).

Discussion

The embryo recovery rate for mares in this study was consistent with that previously reported (Squires et al. 1982, Woods et al. 1986). The pregnancy rates of DAPI-stained embryos and

control embryos were also acceptable when compared with other reported nonsurgical embryo transfers (McKinnon et al. 1989, Pashen et al. 1993).

Staining with DAPI could easily and unambiguously be detected as cells fluoresced either brightly or not at all. Stain uptake of cells along the plane of bisection (Huhtinen et al. 1995) is further evidence for selective uptake of the dye. Moreover, cattle blastomeres lysed by means of bisection are stained with DAPI and propidium iodide in a similar fashion (Bredbacka 1995). Propidium iodide is widely used to selectively stain trophoctoderm cells lysed with immunosurgery.

The embryonic resorption noted here was most likely caused by uterine inflammation of the recipient mare. In our previous study (Huhtinen et al. 1995), embryos were exposed to cytochalasin B and nocodazole in addition to DAPI staining. The reason for early embryonic deaths in that study may not have been caused by DAPI staining, but the combined effect of all treatments may have had adverse effects on the embryo. Since cytochalasin B and nocodazole have not been used extensively in horse embryo experiments, high sensitivity of horse embryos to these compounds cannot be excluded. On the other hand, the rate of embryonic deaths noted in this and our previous study does not significantly differ from the rate of early (2–4 weeks) embryonic losses in commercial brood mares reported by Woods et al. (1985).

The embryos used here were of such good quality that by using morphological criteria or the number of DAPI-stained cells, we could not detect any difference between embryos that survived transfer and those that did not. With the low number of DAPI-stained cells in our study, it is unlikely that a decreased survival rate would be observed with a small number of transfers unless the DAPI treatment itself were clearly toxic to the embryo. With this number of embryos and assuming that the pregnancy rate in the control group were constant, we should have obtained a pregnancy rate in the DAPI-stained group of 2/11 or less to be able to statistically

demonstrate a negative effect of the treatment. The value of a DAPI test could be better demonstrated if embryos with a higher number of lysed cells were transferred. However, the DAPI treatment might then be harmful. The staining procedure most likely facilitates the generation of reactive oxygen species with an adverse impact on non-lysed cells.

Although the low number of embryos is the most common limiting factor in equine embryo research, and almost all embryos in private practice are worth transferring regardless of their quality, situations may arise when a choice has to be made between the embryos to be transferred. In the future, when equine in vitro embryo production technology is further refined and

freezing of embryos becomes routine practice, such a choice might become more realistic. Staining with DAPI might reveal minor quality differences between embryos, since cell death may sometimes be indicative of degeneration of the embryo.

Although the number of embryos available here was insufficient to demonstrate lack of treatment effect, the high pregnancy rates following DAPI and UV treatment should encourage further studies on this subject. As the stained embryos in this study had only a few dead cells (five or fewer), testing the viability of DAPI-stained embryos with a higher number of dead cells would be important.

References

- Bredbacka, P.** 1995. Factors affecting cell viability during bisection of bovine embryos. *Theriogenology* 44: 159-166.
- Brück, I. & Hyland, J.H.** 1991. Measurements of glucose metabolism in single equine embryos during early development. *Journal of Reproduction and Fertility, Supplement* 44: 419-425.
- Huhtinen, M., Bredbacka, P. & Kotilainen, T.** 1995. Non-surgical transfer of DAPI-stained equine demi-embryos treated with cytochalasin B and nocodazole. *Biology of Reproduction, Monograph Series No. 1*: 325-328.
- Hutz, R.J., DeMayo, F.J. & Dukelow, W.R.** 1985. The use of vital dyes to assess embryonic viability in the hamster *Mesocricetus auratus*. *Stain Technology* 60: 163-167.
- Kardymowicz, O.** 1972. A method of vital staining for determining the viability of fertilized sheep ova stored in vitro. In: *Proceedings of the 7th International Congress on Animal Reproduction and Artificial Insemination, München, I, Volume 1*. p. 503-506.
- Low, B.G., Takahashi, Y. & Kanagawa, H.** 1986. Viability of frozen-thawed mouse blastocysts with different numbers of dead cells. *Theriogenology* 25: 675-680.
- Majumdar, A.C.** 1990. Embryo transfer in rabbit: assessment of viability of embryo using dye technique. *Journal of Veterinary Physiology and Allied Science* 9: 15-20.
- McKinnon, A.O., Carnevale, E.M., Squires, E.L., Carney, N.J. & Seidel, G.E., Jr.** 1989. Bisection of equine embryos. *Equine Veterinary Journal, Supplement* 8: 129-133.
- & Squires, E.L.** 1988. Morphologic assessment of the equine embryo. *Journal of American Veterinary Medicine Association* 192: 401-406.
- Mohr, L.R. & Trunson, A.O.** 1980. The use of fluorescein diacetate to assess embryo viability in the mouse. *Journal of Reproduction and Fertility* 58: 189-196.
- Overstrom, E.W., Duby, R.T., Dobrinsky, J. & Roche, J.F.** 1992. Viability and oxidative metabolism of the bovine blastocyst. *Theriogenology* 37: 269. (Abstract).
- Pashen, R.L., Lascombes, F.A. & Darrow, M.D.** 1993. The application of embryo transfer to polo ponies in Argentina. *Equine Veterinary Journal, Supplement* 15: 119-121.
- Petters, R.M. & Lucy, M.C.** 1987. Rhodamine 123 as a cytoplasmic stain for mammalian zygotes. *Theriogenology* 28: 639-646.
- Pruitt, J.A., Forrest, D.W., Burghardt, R.C., Evans, J.W. & Kraemer, D.C.** 1991. Viability and ultrastructure of equine embryos following culture in a static or dynamic system. *Journal of Reproduction and Fertility, Supplement* 44: 405-410.
- , Wilson, J.M. & Kraemer, D.C.** 1988. Viability of equine embryos following fluorescein diacetate staining. *Theriogenology* 29: 291. (Abstract).
- Rieger, D. & Guay, P.** 1988. Measurement of the metabolism of energy substrates in individual bovine blastocysts. *Journal of Reproduction and Fertility* 83: 585-591.
- , Lagneaux-Petit, D. & Palmer, E.** 1987. Preliminary investigations of the metabolic activity of early horse embryos. *Journal of Reproduction and Fertility, Supplement* 35: 699-700.
- Schilling, E. von & Doepke, H.-H.** 1978. A rapid diagnostic test for the viability of early cattle and rabbit embryos using Diacetyl-fluorescein. *Naturwissenschaften* 65: 658.
- , Smidt, D., Sach, B. & El Kaschab, S.** 1979a. Diagnosis of the viability of early bovine embryos by fluorescein diacetate to assess embryo viability in the mouse. *Journal of Reproduction and Fertility* 58: 189-196.

cence microscopy. *Annales de Biologie Animale, Biochimie, Biophysique* 19: 1625–1629.

–, **Niemann, H., Cheng, S.P. & Doepke, H.-H.** 1979b. DAPI – a further fluorescence test for diagnosing the viability of early cow and rabbit embryos. *Zuchthygiene* 14: 170–172.

–, **Niemann, H. & Smidt, D.** 1982. Evaluation of fresh and frozen cattle embryos by fluorescence microscopy. In: Hafez, E.S.E. & Semm, K. (eds.). *In Vitro Fertilization and Transfer*, MTP Press, Falcon House, Lancaster, England. p. 349–355.

Squires, E.L., Imel, K.J., Iuliano, M.F. & Shideler, R.K. 1982. Factors affecting reproductive efficiency in an equine transfer programme. *Journal of Reproduction and Fertility, Supplement* 32: 409–414.

Woods, G.L., Baker, C.B., Hillman, R.B. & Schlafer, D.H. 1985. Recent studies relating to early embryonic death in the mare. *Equine Veterinary Journal, Supplement* 3: 104–107.

–, **Hillman, R.B. & Schlafer, D.H.** 1986. Recovery and evaluation of embryos from normal and infertile mares. *Cornell Veterinarian* 76: 386–394.

SELOSTUS

Hevosen alkoiden värjääminen DAPI:lla ennen vastaanottajaan siirtoa

Mirja Huhtinen ja Peter Bredbacka

Maatalouden tutkimuskeskus

Kokeessa tutkittiin 6–7 päivän ikäisten hevosen alkoiden elävyyttä 4',6'-diamidino-2-phenylindole (DAPI):n avulla. DAPI on fluoresoiva väriaine, joka sitoutuu DNA:han ja värjää alkioista kuolleet solut. Yksitoista alkioita värjättiin 15 minuutin ajan DAPI:lla huoneenlämmössä. Tämän jälkeen värjäys katsottiin UV-valon avulla, minkä jälkeen alkioita pidettiin lämpökaapissa (+37°C, 5% CO₂) kaksi tuntia ennen ei-kirurgista siirtoa vastaanottajattamoihin. Kahdeksan kontrollialkiota käsiteltiin samoin, mutta niitä ei värjätty. Vastaanottajattamoille tehtiin tiineystarkastus ultraäänen avulla kuusi päivää alkion siirron jälkeen, ja siitä lähtien joka toinen päivä

tiineyden keskeyttämiseen asti. Kun sikiön sydämen syke havaittiin, vastaanottajattamoille annettiin prostaglandiinia abortin aikaan saamiseksi. Vastaanottajista joihin siirrettiin DAPI-värjätty alkio tiinehtyi 82 %. Vastaanottajista joihin siirrettiin värjäämätön alkio tiinehtyi 75 %. Yksi DAPI-värjätystä alkioista kuoli vastaanottajattaman kohdussa, eikä alkioita voitu enää havaita 12 päivää siirron jälkeen. Kyseisellä tammalla todettiin seuraavan kiiman aikana tehdyssä tutkimuksessa kohtutulehdus. Vaikka alkoiden lukumäärä kokeessa oli liian pieni, jotta se todistaisi värjäyksen haitattomaksi, koeryhmän korkeasti tiinehtymisprosentti rohkaisee lisätutkimuksiin.