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Dye exclusion technique to assess the viability of pre-implantation bovine embryo based on a murine model

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ABSTRACT

Dye exclusion assay was evaluated for assessing viability of pre-implantation murine and bovine embryos along with morphological examination of sucrose-induced shrinkage and *in vitro* culture. Different concentrations of the dyes were also investigated to determine the optima. A total of 460 murine and 76 bovine embryos, on morphological evaluation revealed 71. 52 and 72.36 per cent viability, respectively. Dye exclusion assay of murine embryos using Trypan Blue (0.05%), Eosin-Y (0.12 mM), Rose Bengal Red (0.5 mM) and Eosin-B (0.12 mM) followed by developmental competence indicated 82.35, 81.25, 78.57 and 50.0 per cent viability, respectively. The corresponding values in case of bovine embyros were 80.0, 87.0, 75.0 and 61.5 percent. The differences in viability estimates due to dyes were statistically significant (P<0.05). Sucrose induced shrinkage of murine and bovine embryos indicated 71.06 and 65.45 per cent viability respectively. Corresponding values by *in vitro* culture were 73.62 and 70.9 per cent. The differences between morphological assay and other methods were significant (P<0.05) but the differences between dye exclusion assay and developmental competence of embryos in culture medium were statistically non significant.

Key words : Viability assay, dye, murine embryo, bovine embryo

Morphological appearance of embryo for viability assessment is a subjective method, requires technical competence. Assessing the embryo viability with the use of vital dyes can be of immense help. Direct assessment of membrane integrity of embryo, the exclusion, uptake and/or retention of an array of vital fluorescent/non-fluorescent probes have been used to correlate membrane integrity with embryo viability *in vitro*. Large molecular weight inert dyes such as Trypan Blue, Rose Bengal Red, etc. can be used to assess membrane integrity as an indirect assay, whereas, fluorescent and non-fluorescent probes have also been used to correlate membrane integrity determien if viability assay of pre-implantation bovine embryos can be carried out efficiently using large molecular weight inert dyes, based upon a murine model. The dye exclusion tests were compared with the routine subjective methods along with evaluation of a proper dye and its optimum concentration for evaluating viability.

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MATERIALS AND METHODS

Experimental animals : Mice, 12-20 weeks of age from Small Animal Colony, PAU, Ludhiana, kept under standard feeding and management conditions were used in the study. Crossbred (Holstein Freisian x Sahiwal x Red Dane) cattle (n = 20) from PAU Dairy Farm, Ludhiana, maintained under general herd management conditions, with good health body condition cycling normally and without any gross reproductive abnormality were taken up for the study. Prior to administration of the superovulatory drug, animals were examined per-rectum for the presence of a corpus luteum.

Superovulation : Mice superovulated using PMSG (Follign, Intervet, International B.V., Boxmeer, Holland) under a standard protocol (Sharma, 1993). Briefly 10 IU of PMSG I.P. followed by 50 IU of chorionic gonadotropin (Chorulon Intervet. International B.V., Boxmeer, Holland) I.P 48 hours later, and introduction of healthy male (1 male with 3 female). The copulation

plugs were confirmed next day morning a .d the female with plugs were used further for flushing o genital tracts. Follicle stimulating hormone (FSH-P, Folltropin-V, 400 mg NIH, Veterpharm Canada Inc., Ontario) was used for induction of superoculation, on day 10-13 of induced/ normal cycle, in cattle. On third day of FSH-P injection, the animals were given a shot of $PGF_2\alpha$ 25 mg. I.M. (Lutalyse Upjohn Limited, U.K.) The animals were inseminated artificially at the onset of standing heat and twelve hourly, till the end of oestrus with frozen semen (Madan *et al.*, 1993).

Embryo collection : The ampullar region of fallopian tube, obtained from mice on fifth day of mating, after dissection was observed under the stereozoom microscope and punctured with sharp needle, leading to release of an opaque white mass of fertilized occytes. The mass of embryos was transferred into holding medium (TCM-199 + 10% FCS + gentamycin 5μg/ml) in 35 mm petridish. The embryos were washed, counted, pooled and transferred to drops of culture medium (TCM-199 + 20% FCS + 0.4% BSA + 5 μ/ml gentamycin) and kept in incubator at 37°C with 5% CO_2 and 95% relative humidity (Sharma, 1993).

Cattle embryos were collected by flushing the animals on day 7 post superovulatory heat using a non-surgical method of collection, by a two way German (Rusch) Catheter, using Dulbecco's-MEM + 0.5% BSA + gentamycin 5 μg/ml, pH 7.2 as flushing medium warmed to 37°C prior to use. Embryos were transferred into a small falcon dish (35 mm dia) containing holding medium. Embryos recovered were ascribed to various stages according to the classification proposed by Linder and Wright (1993) as morula (M), compact morula (CM), early blastocyst (EB), explanded blastocyst (E x B) and hatched blastocyst (H x B).

Viability assays

a. Morphological : A single, non-invasive rapid procedure of microscopic examination as a gross indicator of embryo health/viability (Shea, 1981; Linder and Wright, 1983) was carried out on the basis of embryo colour/darkness, homogeneity of blastomere size, cytoplasm granulation and degree of blastomere fragmentation.

b. Sucrose induced shrinkage ; Sucrose (1M) induced shrinkage of the viable embryonic cell was within one minute of exposure to 75 per cent of original volume was used as indicator of embryo viability. Embryos not undergoing shrinkage within 2 to 3 minutes and lacking reorientation (95% volume) were graded doubtful (Yang *et al.*, 1988; Vansoom *et al.*, 1996).

c. *In vitro* culture (IVC) : Embryos flushed, graded and classified into various stages were cultured in 50μl drops of embryo culture medium (TCM-199 + 20% FCS + 5% BSA) under light liquid paraffin at 37.6°C, 5% CO_2 and 95% relative humidity. Embryos developing to next developmental stage were considered viable.

d. Dye exclusion assay : A fixed number of embryos of different developmental stages were exposed to different water soluble dyes, viz. Eosin Yellow (Eosin Y), Eoxin Blue (Eosin B), Trypan Blue (Trypan B) and Rose Bengal Red (Rose Bengal R) at different concentrations as tabulated below :

S.No.	Dye	Concentrations			
1.	Eosin Y (mM)	0.06	0.12	0.60	1.20
2.	Eoxin B (mM)	0.06	0.12	0.60	1.20
3.	Trypan B (%)	0.025	0.05	0.075	0.10
4.	Rose Bengal R (mM)	0.1	0.5	0.75	1.0

The embryos were exposed for one to two minutes per concentration and washed twice in Dulbecco's-MEM to eliminate the excess stain. Embryo stained and partially stained were considered non-viable. The embryos exposed to dye were incubated within two hours of collection, assessed after 24 hours and compared with the stage of development recorded immediately after collection. Embryos with signs of degeneration of no development were considered to be non-viable and those at next stage after 24 hrs of IVC as viable.

e. Statistical methods : The data generated from the above trials was statistically analysed as described by Snedecor and Cochran (1967). Briefly, the percent values were transformed to Arc Sine and subjected to ANOVA and significant differences were compared by critical diferences (least significant differences).

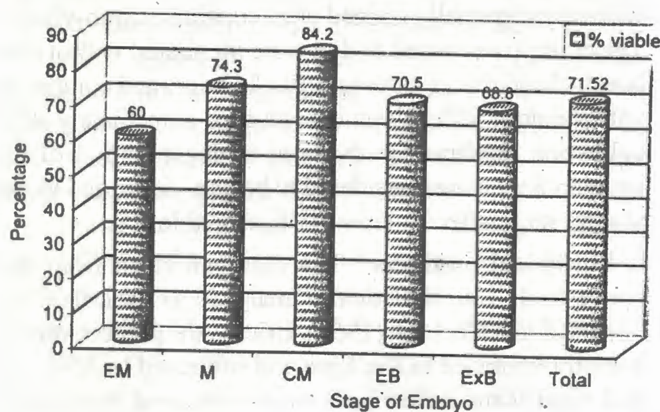
RESULTS AND DISSCUSSION

a. Morphological evaluation : Out of the 460 murine embryos collected, 71.52 per cent (329) were judged

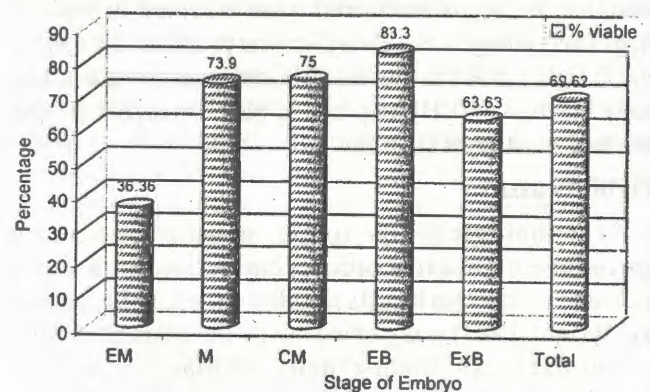
Table 1. Dye exclusion and developmental competence assays of morphologically viable murine embryo

Dye	Concentration	Morphological viable	Dye Exlcusion		Developmental competence	
			Viable		Viable	
		No.	No.	%	No.	%
Eosin Y (mM)	0.06	24	22	91.6	21	95.4[a]
	0.12	18	16	88.8	13	81.25[a]
	0.6	31	28	90.32	19	67.85[b]
	1.20	21	18	85.7	9	44.4[b]
Total		**94**	**84**	**89.36**	**61**	**72.6**
Eosin-B (mM)	0.06	26	23	88.46	15	65.21[a]
	0.12	32	30	93.75	15	50.0[a]
	0.6	19	17	89.47	7	41.17[b]
	1.20	18	15	83.33	5	33.33[b]
Total		**95**	**85**	**89.47**	**42**	**49.41**
Trypan Blue (%)	0.025	13	11	84.6	9	81.81
	0.05	19	17	89.47	14	82.35
	0.75	15	12	80	9	77.77
	0.10	13	11	84.61	9	81.81
Total		**60**	**51**	**85.0**	**41**	**80.39**
Rose Bengal Red (mM)	0.1	15	13	86.65	10	76.92
	0.5	30	28	93.33	22	78.57
	0.75	13	12	92.30	8	66.66
	1.0	22	20	90.09	15	75.0
Total		**80**	**73**	**91.25**	**55**	**75.34**
Overall Total		**329**	**293**	**89.05**	**199**	**67.90**

– Figures with different superscripts a & b ($P<0.05$) vary significantly compared to the lowest concentration.
– Effectiveness of dye evaluated at $P<0.05$ in increasing order.
Eosin B < Rose Bengal Red < Eosin Y < Trypan Blue.



1a : Murine embryos



1b : Bovine embryos

Fig. 1(a&b). Morphological evaluation of embryos at various developmental stages

Table 2. Dye exclusion and developmental competence of morphological viable bovine embryos

Dye/ Concentration	n	Morphology		Dye Exclusion		Developmental competence after dye exclusion	
		Viable		Viable		Viable	
		No.	%	No.	%	No.	%
Eosin Y 0.12 mM	15	11	73	8	72.7	7	87.5
Eosin B 0.12 mM	24	18	75	13	72.2	8	61.5
Trypan Blue 0.05%	20	15	75	10	66.6	8	80.0
Rose Bengal Red 0.5 mM	17	11	64.7	8	72.7	6	75.0
Total	**76**	**55**	**72.36^{a}**	**39**	**70.09^{b}**	**29**	**74.38^{b}**

Figures with different superscripts vary significantly ($P<0.05$).

viable on morphological evaluation (EM, 60%; M, 74.3% CM, 84.2%, EB, 70.5% and ExB, 68.8%; Table 1 and Fig. 1a). In bovine out of 76 embryos 72.36 per cent (55) were observed live (EM, 36.36%; M, 73.9%; CM, 75.0%.; EB, 83.3% and ExB, 63.63%; Table 2 and Fig. 1b).

The overall percent murine and bovine live embryos recovered were 71.52 and 69.92 respectively. The results obtained in our study were higher than the findings of Schilling *et al.* (1980) who reported a recovery of 51.5 per cent normal embryos. However, Nakagawa *et al.* (1991) have reported a high percentage of 89.27.

b. Sucrose induced shrinkage : Out of 273 murine embryos, 71.06 per cent (EM, 70%; M, 77.06%; CM, 71.66%; EB, 63.63% and ExB, 55%) were found viable by subjecting them to sucrose induced shrinkage, whereas, in bovine 65.54 per cent (EM, 75; M, 64.7%; CM, 75%, EB, 60% and ExB, 57.14%) embryos, out of 55 exhibited shrinkage on sucrose treatment.

Since work regarding this aspect on embryo viability assays has not been reported so far except some indirect indications regarding shrinkage of cellular contents in case of zygotes/embryos to increase peri-vitelline space for sperm injection procedures. So our findings can be taken as a prelude to evaluate this test by repeated trials on larger number of embryos.

The differences between developmental stages were not statistically significant but the values were somewhat higher for EM and CM in cattle. This may be due to adequate shrinkage and better visualization of cell parameters during these development stages.

c. *In vitro* culture : Progressive development was seen in 73.6 per cent (201) out of 273 murine embryos (EM, 72.5%; M, 77.98%; CM, 71.6%; EB, 72.7%; ExB, 60%) after subjecting to IVC. In case of bovine embryos IVC revealed a viability of 70.0 per cent (39 out of 55) embryos subjected to 24 hours of IVC (EM, 75%; M, 64.7%; CM, 83.3%; EB, 73.3%; ExB, 57.14%).

Present investigation revealed a developmental competence of 73.62 and 70 per cent for murine and bovine embryos, respectively, while Ridha and Dukelow (1985) reported 81 per cent for hamster embryos. The results were better for M, CM and EB stages in murine and EM and CM in cattle, though they did not differ significantly. The results of the present study were almost similar to Xu *et al.* (1992) who reported 65.9 per cent developmental competence for zygotes and slightly higher percentage may be due to screening out of the embryos of early stages.

Miyoshi *et al.* (1997) reported a stage dependent development of murine embryos in a chemically defined medium from one-cell stage to blastocyst formation as

54.5 per cent. The rate of blastocyst formation was higher due to the reason that only morphologically graded viable embryos were used for IVC.

d. Dye exclusion assay : Per cent viable murine and bovine pre-implantation embryos subjected to dye exclusion assays are presented in tables 1 & 2.

1. Eosin-Y : Eosin- Y Dye exclusion assay as a tool for viability assessment has not been reported in literature so far. Sixty one (72.6%) embryos were observed live when 84 embryos (that were morphologically categorized live out of 94) were subjected to Eosin-Y Dye exclusion assay. Significant difference ($P<0.05$) between morphological and stain assay for viability evaluation, indicated that judgement on morphological basis may be subjective and limited to the discrimination of degenerated embryos and preclude morphological criteria as an accurate measure of embryo develomental potential *in vivo*. No significant difference between Eosin-Y dye exclusion assay and developmental competence of the preimplantation embryo was observed.

In bovine, the viability assessed with Eosin-Y dye exlcusion assay was 72.7 per cent out of which only 85.5 per cent developed to the next developmental stage. The results suggest that at concentration $\leq$0.12 mM of Eosin-Y was not toxic, since higher per cent of embryos developed to the next developmental stage.

2. Eosin-B : Results observed in the present study (49.41%) are similar to those observed by Dooley *et al.* (1984) who reported that the per cent embryos developing to blastocyst following treatment were 51 per cent for the dye concentration of 0.12 mM as compared to 45 per cent for the control. In case of bovine embryos, no discrepancy between dye exclusion assay and developmental competence was evident but a significant difference ($P<0.05$) between morphology and dye exclusion assay was observed. The results indicate that Eosin B dye concentration below 0.12 mM were nontoxic to embryo development and thus an assay based on Eosin-B dye exclusion can be developed.

3. Trypan blue : Trypan B dye at a concentration of 0.05 per cent in modified Whitten's medium has been used to evaluate toxicity of this vital stain for frozen thawed murine embryos flushed at blastocyst stage, by exposure to the dye and cultured till the next developmental stage. The present study elucidated no significant difference between dye exclusion and developmental competence by *in vitro* culture of murine embryos. However, significant difference ($P<0.05$) between morphological and dye exclusion assays was evident. No significant difference between stained (17.2%) and unstained (12.3%) groups was observed by Thandhi *et al.* (1982). Similarly, Majumdar (1990) reported no teratogenic effect of dye on transfer of rabbit embryos.

In cattle embryos there was a significant difference ($P<0.05$) between the morphological evaluation and dye exclusion assay, indicating that morphological assessment of embryo viability to be less accurate and needs assessment with alternate methods.

4. Rose Bengal Red : Rose Benal-R strain at a concentration of 3 per cent has been used for sperm evaluation, but its use in embryos viability assay has not been reported so far. There was no significant difference between unstained embryos and their development competence by *in vitro* culture, however, there was a significant differeence ($P<0.05$) of dye exclusion assay compared to morphological evaluation of murine and bovine embryo viability. These observations lead us to infer that Rose Bengal R at 0.5 mM concentration may be used to assess the bovine embryo viability.

The present study indicated that the dye exclusion assay along with subjective morphological evaluation may be employed for a fairly accurate predictability regarding embryo viability. It further revealed that optimum concentration for Trypan-blue, Eoxin-Yellow, Rose Bengal Red and Eosin-Blue (all written in order of merit) were 0.05 per cent, 0.12 mM, 0.05 mM and 0.12 mM, respectively.

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