

PENGGUNAAN BERBAGAI CARA PENGECATAN UNTUK EVALUASI PERKEMBANGAN DAN VIABILITAS EMBRIO SAPI PEROLEHAN *IN VIVO* DAN *IN VITRO*

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Abstrak

Embrio transfer memerlukan embrio yang bermutu baik. Penelitian ini merupakan suatu usaha untuk mendapatkan metoda yang baik dalam mengevaluasi embrio. Dalam penelitian ini embrio hasil fertilisasi *in vitro* diklasifikasikan ke dalam freezable dan non-freezable *in vitro* secara subyektif. Melalui pengecatan Giemsa embrio tersebut dibandingkan dengan embrio hasil fertilisasi *in vivo* yang freezable. Perkembangan dan viabilitas embrio dievaluasi menggunakan cat fluorescent (FDA dan Hoechst 33342) kemudian dibandingkan dengan hasil setelah pengecatan Giemsa.

Jumlah sel dalam embrio hasil fertilisasi *in vitro* dan *in vivo* yang freezable tidak berbeda ($P > 0,1$), sedangkan pada embrio hasil fertilisasi *in vitro* yang freezable dan non-freezable menunjukkan adanya perbedaan yang signifikan ($P < 0,001$) dalam hal jumlah sel.

Kesesuaian antara H 33342 dan cat Giesca cukup tinggi (63%) pada embrio yang memiliki jumlah sel kurang dari 20, dan rendah (25 - 34 %) pada embrio yang memiliki jumlah sel lebih dari 20. Terdapat korelasi yang signifikan (0,82) antara tingkat fluoresensi dari cat FDA dan jumlah sel pada pengecatan Giemsa.

Klasifikasi subyektif pada embrio hasil fertilisasi *in vitro* dapat digunakan secara praktis. Pengecatan FDA merupakan alat yang baik untuk klasifikasi tersebut sekaligus untuk mengevaluasi perkembangan dan viabilitas embrio pada berbagai tahap yang berbeda. Hoechst 33342 dapat sebagai metode yang berharga untuk mengevaluasi perkembangan embrio pada fase awal.

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THE USE OF DIFFERENT STAINING METHODS TO EVALUATE THE DEVELOPMENT AND VIABILITY OF BOVINE EMBRYOS OBTAINED *IN VIVO* AND *IN VITRO*

Abstract

In an attempt to find a good method to evaluate embryos we classified the *in vitro* embryos into freezable and non-freezable by subjective method. These embryos were compared with *in vivo* freezable embryos using Giemsa staining. The embryo development and viability was evaluated by fluorescent stain (FDA and Hoechst 33342) and compared with the result after Giemsa stain.

That number of cells in freezable *in vitro* and *in vivo* embryos were not different ($P > 0.1$), while *in vitro* freezable and non-freezable embryos showed significantly differences ($P < 0,001$) in number of cells.

The agreement between Hoechst 33342 and Giemsa stain was quite high (63%) in embryos with number of cells less than 20, and low agreement (25 - 34 %) in embryos with number of cells more than 20. There was a significantly correlation (0.82) between the degree of fluorescent of FDA stain and the number of cells at Giemsa stain.

The subjective classification of *in vitro* embryos can be used in the practical situation. FDA staining is a good tool to check this classification as well as to evaluate the development and viability of embryos in different steps of experimental trials. Hoechst can be a valuable method to evaluate the development of early stage embryos.

Introduction

Bovine embryo transfer needs supply of good quality embryos, which can be obtained from superovulated and inseminated cows or after *in vitro* fertilization. The evaluation of the embryo viability by morphological examination provides useful trends but is not specific enough to assess the potential viability of embryos and is very subjective. The most conclusive method for assessing the influence of different procedures on

the viability of embryos is to monitor embryonic development (Pursel *et al.*, 1985b). It would be useful to be able to evaluate the quality of embryos more objective.

Different staining method has been used for evaluation of embryo viability and development. Fluorescein diacetate (FDA), fluorescent vital staining, was used to evaluate embryo viability by measuring the degree of fluorescence (Rotman and Papermaster, 1966; chilling *et al.*, 1979; Morh and Trounson, 1980; Niemann, 1980; Hoppe and Bavister, 1984). Hoechst 33342 DNA stain has been described as a method for rapid assessment of the number of cells in bovine embryo (Pursel *et al.*, 1985a; Ellington *et al.*, 1990; Viuff *et al.*, 1991). The staining also has been used to determine the total number of blastomeres, the number of poor nuclei and the number of nuclei in mitosis (Ellington *et al.*, 1990).

Giemsa staining had been described as a simple technique for making chromosome preparation from zygotes and early blastocysts (King *et al.*, 1979).

The aims this study were 1) to classify the *in vitro* obtained embryos into two categories according a subjective method and to stain with Giemsa to check the accuracy of the classification 2) to analyze the capability of FDA and Hoechst 33342 stains to estimate the development and viability of *in vitro* and *in vivo* embryos and to compare the result with the number of cells and pycnotic and mitotic cells of the embryos at Giemsa staining.

Materials and Methods

The embryos used, morulae and blastocyst, were obtained either from superovulated cows, or from *in vitro* fertilization.

Superovulations were induced by giving follicle stimulating hormone (FolltropinR, Vetrepharm, Canada) intra muscularly twice daily for four days (total dosage 28 mg). Luteolysis was induced with a single dose of cloprostenol (EstrumateR, Cooper, U.K.) on day 3 of FSH treatment. Embryo collection were done non-surgically seven days following artificial insemination.

The *in vitro* embryos were obtained from *in vitro* culture (conditioned medium) of *in vitro* fertilized *in vitro* matured oocytes.

Excellent and good quality embryos are routinely frozen in this laboratory and are called freezable in this study whereas the fair and poor quality were considered as non-freezable. The classification was based on appearance of embryos, compactness of the cells and development stage (IETS Manual, 1987).

In vitro and *in vivo* obtained embryos were divided randomly into 2 groups as follows:

1) *In vivo* and *in vitro* embryos for treatment with FDA, then Giemsa. The embryos were divided into subgroups according to different degree of fluorescence at FDA staining and the correlation of these subgroups with the number of cells at Giemsa staining were checked.

2) *In vivo* and *in vitro* embryos for treatment with Hoechst 33342, then Giemsa. The embryos were divided into three subgroups according to the number of cells at Giemsa staining and the agreement with the number of cells counted at Hoechst 33342 was calculated.

All the data were analyzed statistically by either student's t-test or non-parametric correlation test.

The assay methods

1. FDA assay

A stock solution of FDA (Sigma) was prepared by dissolving FDA (5 mg/ml) in acetone. The final working solution was made by adding 0,5 ul of stock FDA solution to 3 ml of PBS. Embryos were incubated in the solution for 1 minute, rinsed twice in fresh medium (PBS) (1 minute each rinse) and then immediately exposed to UV-light. The parameter used was the brief percentage of the embryo that gave fluorescence (0-25, 26-50, 51-75, 76-100 %). The observations were made with an Leitz-Dialux 20 microscope fitted with UV-light.

2. Hoechst assay

A stock solution of Hoechst 33342 was prepared by dissolving Hoechst 33342 (1 mg/ml) in PBS, and stored at 4°C without exposure to light. Just prior to use, a working solution (5 ug Hoechst 33342 in 3 ml PBS) was prepared. Embryos were cultured in the working solution of Hoechst 33342 for 30 minute at 39°C in 5% CO₂ in air at high humidity. The fluorescence evaluation were performed by exposing it to UV light. The parameter used was the number of cells that give fluorescence.

3. Giemsa assay

Embryos were treated with hypotonic solution (1% sodium citrate solution) for five minutes. The fixation was done in two steps. First fixation (quick and dispersing the cells by blowing over the slide) was methanol: acetic acid (1:1, v/v). Second fixation was in methanol: acetic acid (3:1, v/v) for at least four hours but not more than over night. After that embryos were stained with 0.5 % Giemsa and evaluated under the microscope (King *et al*, 1979). The parameter used were total number of cells and pycnotic and mitotic index which were made by dividing the number of pycnotic and mitotic cells with total number of cells.

Results

The selected freezable *in vitro* embryos did not differ significantly ($P > 0.1$) in number of cells from freezable *in vivo* embryos. The comparison between freezable and non-freezable *in vitro* embryos shown significantly different ($P < 0.001$) in number of cells at Giemsa staining.

Significant differences were found on pycnotic index between *in vivo* freezable and *in vitro* non-freezable embryos ($P < 0.1$). (Table 1)

Table 1. Comparison between freezable, non-freezable, *in vitro* and freezable *in vivo* embryos using Giemsa staining method.

	Number of cells	Mitotic index	Pycnotic index
<i>In vivo</i> (n=12) freezable	72,8 (65,3-80,3)	0,05 (0,04-0,06)	0,13 (0,09-0,17)
<i>In vitro</i> (n=11) freezable	57,8 (49,0-66,0)	0,035 (0,03-0,04)	0,19 (0,12-0,26)
<i>In vitro</i> (n=14) non-freezable	21,9 (18,0-23,0)	0,2 (0,14-0,27)	0,29 (0,22-0,36)

The agreement in number of cells between Hoechst and Giemsa was quite high (63%) in embryos with less than 20 cells, but lower in embryos with more than 20 cells (25 to 34%) (Table 2)

Table 2. Agreement in number of cells/embryo comparing Hoechst 33342 and Giemsa staining methods.

Number of cells (Giemsa)	Number of cells (Hoescht)	Agreement(%)
≤ 20 (n=6)	10.6 (9.4-11.8)	63.33 ± 16.4
21-50 (n=6)	11.7(10.2-13.2)	34.1 ± 3.8
≥ 51 (n=7)	21.2(18.7-23.7)	25,6 ± 4.0

There was a high correlation (0,82, Spearman's correlation test, P<0,002) between the degree of fluorescence at FDA staining method and the number of cells at Giemsa stain (Table 3). There was a tendency towards negative correlation (-0,37) between FDA staining and number of pycnotic cells. The pycnotic index increased when the proportion of fluorescence decreased.

Table 3. Correlation between percentage of fluorescence at FDA staining and total number of cells at Giemsa staining on bovine embryos.

%of fluorescence (FDA)	Number of cells (Girma)	Pycnotic index
0 - 25 (n = 3)	13.7 ± 5.3	0.28 (0.09 - 0.47)
26 - 50 (n = 4)	17.5 ± 4.5	0.19 (0.08 - 0.3)
51 - 75 (n = 3)	17.0 ± 5.0	0.12 (0.01 - 0.23)
76 - 100 (n = 8)	70.4 ± 7.4	0.05 (0.04 - 0.06)

Discussion

In vitro production of embryos appears to have good potential and future uptake of the technology is likely to be significant (Gordon and Lu, 1990). It was proposed that the reduced proportion of the ICM expresses a reduced viability (Wiolladsen and Polge, 1981) and may be the cause of the low pregnancy rates of blastocysts derived from *in vitro* fertilization

(Iwasaki *et al*, 1990). To classify the *in vitro* obtained embryos by subjective method using compactness, appearance and development of the embryos and divide those embryos in freezable and non-freezable was useful in this experiment. Freezable *in vitro* embryos were morphologically similar to *in vivo* produced embryos, since there were no significant differences in number of cells and pycnotic index between the two groups at Giemsa staining. In addition the difference in development between freezable and non-freezable *in vitro* embryos was highly significant, freezable embryos had more cells number.

Morphological criteria alone has been discussed as a good indicator of potential viability of the embryos (Shea *et al*, 1976). FDA and Hoescht 33342 DNA stains has been used to find an easy and non-toxic assay for embryo viability.

The Hoescht 33342, which is a bisbenzimidazole fluorochrome that binds reversibly to sequences of three or more adenine-thymine pair of double stranded DNA, has been used for visualization of chromatin in oocyte, zygotes, and embryos in several species, including cattle (Pursel *et al*, 1985a; Critser and First, 1986).

Ellington *et al.* (1990) reported that Hoescht 33342 stain was useful to characterize the normal appearance of day 6 embryos developed *in vivo*. The embryos were placed on a siliconized glass microscope slide, covered by the stain and coverslip, requiring light pressure on the coverslip to cause embryos flattening for balastomere assessment. Embryo stained in this manner remain suitable for evaluation for more than a year if kept in a light-proof container.

Viuff *et al.* (1990) reported that the agreement in Hoescht 33342 and aceto-orcein stains following oocyte maturation or fertilization *in vitro* (up to 2 cell stage) was 74%. With the method used in this study, the average agreement between Hoescht 33342 and Giemsa stain was approximately 40%.

When the embryo had small number of cells, up to 20 cells, the correlation was higher (63.33%). Other studies using early stage embryos and flattened embryos found higher agreement, while in our study, the poor result by looking at 7 days embryos in a petri dish are because the cells masking each other and therefore made it difficult to count accurately.

FDA is used to assess the viability of cells lines cultured *in vitro*. Living mammalian cells accumulate intracellular fluorescein when exposed to FDA (fluorochromasia). The hydrolysis of fluorescein diacetate by esterase enzyme activity and membrane integrity result in the accumulation of intracellular fluorescein. This stain was found non-toxic to mammalian cell lines in culture (Rotman and Papermaster, 1966; Niemann, 1980; Mohr and Trounson, 1980). Furthermore procedures used in the FDA viability assay were not detrimental to development of late cleavage stage mammalian embryos and thus seem suitable for rapid screening of manipulated embryos for potential damage (Schilling *et al.*, 1979 Hoppe and Bavister, 1984). Embryos with normal stage of development have show repeatable accumulation of intracellular fluorescein FDA stain (Church and Raines, 1980). Mohr and Trounson, 1980). No false negative result were obtained using FDA (embryos that developed but failed to fluoresce) (Hoppe and Bavister, 1984). In addition, Niemann (1980) and Schilling *et al.*, (1979) reported that high percentage of the embryos (85%) showed mitosis after culture when FDA was positive and no development of the embryos when FDA was negative.

There was a high correlation (0.82) between the percentage of fluorescent in FDA stain and the total number of cells in Giemsa stain in

this study, and negative correlation(- 0.37) between percentage of fluorescence in FDA and number of pycnotic cells. It's explained by the presence of a major number of living cells in those embryos with high fluorescence and the increasing of pycnotic cells number in those embryos with low fluorescence. *In vitro* embryos that did not develop after culture showed less fluorescence because they have lower number of cells and cells that lost their viability. It's supported by Schilling *et al.* (1979) who reported, no fluorescence in unfertilized eggs and slowly developing of bovine embryos (4-16 cells) recovered between 5 to 9 days after breeding. Furthermore, embryo with partial fluorescence retained a poor capacity for development (Niemann, 1980). High correlation (0.96) was found between the ability of the embryos to fluorescein and their ability to develop in culture after freezing and thawing (Jackowski, 1977 cited by Mohr and Trounson, 1980).

The fluorescence of the embryos in a few cases became fade very rapidly (3 to 5minutes). We suggest that it could be produced by damage on the membrane of embryos during pipeting . Mohr and Trounson, (1989) reported that normally the fluorescence is kept for half to one hour and they pointed out that some embryos that accumulated intracellular fluorescein were damaged by piercing the membrane, and all accumulated fluorescein was lost.

Conclusion

The common microscopic examination and classification of *in vitro* embryos using subjective parameters, as it is done with *in vivo* embryos, can be used, in the practical situation.

Despite of after staining with Giemsa the embryos are not able to use, this stain is an accurate method to evaluate embryo quality and development, counting the number of cells, pycnotic and mitotic cells. The stain was useful to test the result of fluorescent staining.

FDA staining method can be a valuable tool to evaluate embryo development and viability, as well as to check subjective classification during experimental trial of bovine embryos. Hoescht 33342 staining method can be a practical and useful test to evaluate the development of early stage embryos.

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