

Amniotic fluid cytology at parturition from Nelore calves conceived by artificial insemination, embryo transfer, or in vitro production

C.F. Moya^{1,3}, N.C. Prestes¹, M. Piagentini¹, N.S. Rocha²

¹Department of Animal Reproduction and Veterinary Radiology.

²Department of Veterinary Clinics of the FMVZ/UNESP, CEP 18618-000, Botucatu, SP, Brazil.

Abstract

The aim of the present study was to describe the differences in the cytological pattern of amniotic fluid at the moment of parturition, comparing the data from Nelore calves conceived by *in vitro* production, conventional embryo transfer, or artificial insemination. Sixty Nelore cows were divided in three groups as follows: Group 1, 20 pregnant cows carrying Nelore calves conceived by *in vitro* production after follicular aspiration; Group 2, 20 pregnant cows carrying Nelore calves conceived by superovulation of embryo donor cows; Group 3, 20 pregnant Nelore cows carrying calves conceived by artificial insemination. Near to the time of parturition, cows were transferred to a maternity paddock, to allow for observation of parturition. During the expulsion phase, the amnion was punctured and 15 ml of fluid were collected in plastic vials and frozen at -18°C for later analysis. After thawing, the samples were centrifuged and submitted to Hematoxylin-Shorr staining; the glass slides were covered with Canadian Balsam and a cover slip for further evaluation. Epidermal maturity was determined based on the percentage of keratinized cells stained with Hematoxylin-Shorr. Basal and parabasal cells were not identified on the slides from analyzed amniotic fluid samples. The small intermediate cells were characterized by either an oval shape or a polygonal form with a large cytoplasm and central nucleus. The large intermediate cells had a central nucleus and a higher nucleus: cytoplasm ratio when compared to the superficial cells, which were the largest cells observed. The superficial cells were keratinized cells with angular edges, with either a pyknotic nucleus or no nucleus. There were no differences ($P > 0.05$) among groups when epidermal maturity was analyzed with the Hematoxylin-Shorr staining, thus indicating absence of differences in epidermal development among calves produced by IVP, ET, or AI.

Keywords: amniotic fluid, cytology, bovine.

Introduction

The Brazilian beef cattle industry has grown during the past 20 years as a result of reproductive and production efficiency associated with the advance in the

pressure of breed selection. Breeding and selection has been primarily performed by artificial insemination (AI) and progeny tests followed by embryo transfer (ET). An even greater increase has been obtained by the development of *in vitro* embryo production techniques (IVP) and their incorporation into the production sector.

The facilitation of genetic improvement by introducing desirable traits makes embryo transfer an essential tool for a more rapid and precise animal selection process (Reichenbach *et al.*, 2002). However, the *in vitro* production of embryos as part of commercial programs has overcome the actual indexes acquired by classic embryo transfer in respect to the number of calves produced per cow per year. In addition, this technique results in the use of animals with acquired fertility problems (Tervit, 1996; Goodhand *et al.*, 1999; Malard *et al.*, 1999; Taneja *et al.*, 2000). The large-scale use of this technique results in the occurrence of disturbances at the end of gestation, which have resulted in calves born with higher birth weights than normal (Large Offspring Syndrome), prolonged gestation, high incidence of abortion, premature parturition, high indexes of neonatal mortality, and increased genetic abnormalities (Leibfried-Rutledge, 1999; Wagtendonk-Deleeuw *et al.*, 2000; Prestes, 2005). These possible abnormalities deserve more refined studies describing the aspects involved during those events. The amniotic fluid is an important indicator of fetal health among the factors that can help clarify problems. Amniotic fluid can be submitted to a variety of tests involving biochemistry, cytology, biophysics, and immunology. These evaluations can determine the maturity of pulmonary, renal, and epidermal fetal systems. In addition, they can possibly detect genetic abnormalities and other diseases. The cytology examination of amniotic fluid can help in diagnosing the ruptured membranes and chorio-amnionitis in addition to determining fetal maturity, prenatal sex, and neural tube defects (Kjeldsberg and Knight, 1993).

Cytological studies in humans have described in great detail the types of cells found in the amniotic fluid. The main sources of exfoliated cells in the fluid are the fetus and the amnion. The squamous epithelial cells include the anucleate, superficial, intermediate, and parabasal types (Schrage *et al.*, 1982). Two studies have determined fetal maturity in bitches submitted to cesarean section by the use of Hematoxylin-Shorr stain

³Corresponding author: carlafredrichsen@yahoo.com.br

Received: July 12, 2006

Accepted: February 14, 2007

and classifying the amniotic fluid cells as four types: I - immature or deep cells; II - mature or intermediate cells; III - mature cornified cells and nucleated or superficial cells; and IV - hyper-mature cornified and anuclear cells or squamous cells (Barreto, 2002; Martins and Prestes, 2003). These authors concluded that the fetus was in a post-maturity stage when the percentages of the squamous cells were greater than 90%.

The aim of the present study was to classify and describe possible differences in amniotic fluid cytology and epidermal maturity at parturition. Comparisons were made among results obtained from Nelore calves conceived by embryo transfer, *in vitro* production, or artificial insemination in an attempt to clarify the several problems observed during research and in field conditions in commercial programs. The present study was designed considering the inconsistent results regarding the incidence of fetal problems at parturition and due to the few studies determining the degree of fetal maturity by typifying amniotic fluid cytology in different embryo production systems.

Materials and Methods

This study was performed at the Department of Animal Reproduction and Veterinary Radiology in collaboration with the Veterinary Pathology Service at the College of Veterinary Medicine and Animal Sciences, Botucatu, Brazil (22°51'S/48°26'W). Sixty animals were used, 20 pure bred Nelore cows and 40 embryo recipient cows of mixed breeding and provided by private farms and located in Avaré, Brazil (23°03'S/48°55'W). Herd health and vaccination programs were performed according to the farm management schedule. The animals were divided into three groups: Group 1, 20 pregnant recipient cows carrying pure bred Nelore calves conceived by inoovulation of IVP embryos from donors submitted to follicular aspiration (performed by Bioembryo®, a private company); Group 2, 20 pregnant recipient cows carrying purebred Nelore calves conceived by superovulation of donor cows (ET performed by Tecgene®, a private company); and Group 3, 20 purebred Nelore pregnant cows previously submitted to artificial insemination by a farm technician (control group).

All animals were maintained on pasture and supplemented with silage, concentrate, and water *ad libitum*. All 60 animals from the three experimental groups were transferred to a maternity pasture close to the expected calving date for better observation. The amniotic fluid aspiration was performed during the expulsion phase, after disruption of the chorioallantois. A volume of 15 ml of amniotic fluid was collected using a 40 x 12g needle and a 20 ml syringe. The samples were placed in 15 ml plastic vials and frozen at -18°C for later analysis. The samples were thawed in the laboratory inside a plastic recipient containing water

and ice at an environmental temperature of 24°C. After thawing, the samples were placed in a refrigerated centrifuge at -4°C and 2683 x g for 60 min to separate the excess mucous present in the samples. After centrifugation, 5 ml of each sample was taken and processed on a slide for cytology examination after being stained with Hematoxilin-Shorr. Due to the low cell content in the aspirated fetal fluids, the samples were taken to the Veterinary Pathology Laboratory and centrifuged once again at 2683 x g for 6 min and slides were prepared and stained with Hematoxilin-Shorr (Arruda *et al.*, 1976; modified by Oliveira *et al.*, 2000). At the end of the described procedure, the stained slides were prepared in duplicate, and a Canadian balsam with a cover slip were applied to them. After being air dried, slides were submitted to a light microscope evaluation at 200x and 400x to verify the morphology and staining characteristics of the fetal epithelial cells. The cell classification was based on the maturation stage in relation to its staining affinity. The hematoxilin stains preferably the DNA, RNA, nuclei, and the cytoplasm regions rich in ribosomes and it is represented by a blue color (Gartner and Hiatt, 2003). Otherwise, Shorr staining is specific for the cytoplasm and the keratinized elements, which stain red/orange while the non-keratinized elements stain in green (Oliveira *et al.*, 2000). The fetal epithelial cells were classified in four types: small intermediate cells (SIC), large intermediate cells (LIC), nucleated superficial cells (NSC), and anuclear superficial cell (ASC). Due to the use of Hematoxilin-Shorr staining, the immature (intermediate) cells were stained in green and the mature (superficial) cells were stained in orange. The stain allowed for detailed visualization of nuclei and the content of the cytoplasm.

One hundred cells were counted on each slide for a total of 200 cells per sample. Fetal maturity was evaluated by counting the mature or keratinized cells (KC) in the amniotic fluid, which was performed after the cell classification previously described. Percentage of different cell types including keratinized cells were statistically compared by the Chi-square test with significance level at $P < 0.05$.

Results

The amniotic fluid samples submitted to analysis did not show the presence of either basal or parabasal cells in Groups 1, 2, and 3. The small intermediate cells were observed in all groups and had an oval or polygonal form, characterized by a large cytoplasm and central nucleus (Fig. 1A). The intermediate large cells had a central nucleus and a higher nucleus:cytoplasm ratio when compared to the superficial cells (Fig. 1B), which were the largest cells observed. They were keratinized with angular edges and either a pyknotic nucleus (Fig. 1C) or no nucleus (squamous cells; Fig. 1D). The cell types were divided

in two groups: the immature cells (SIC and LIC) and mature cells or keratinized cells (SNC and ASC). Mature cells were found in larger amounts in the

analyzed amniotic fluid samples. The cytology results from different types of cells stained with Hematoxylin-Shorr are presented in Table 1.

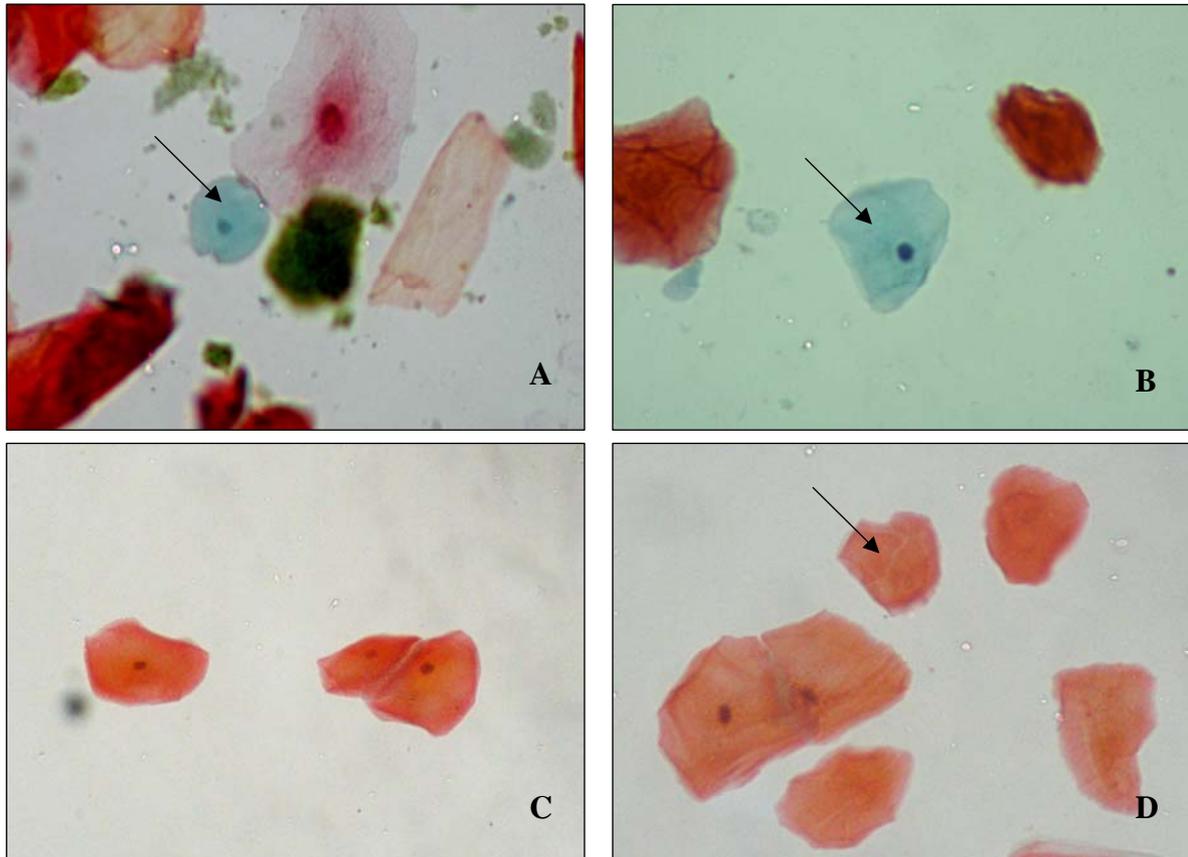


Figure 1. Photomicrography of a small intermediate cell (A, arrow), large intermediate cell (B, arrow), nucleated superficial cells (C), and anuclear superficial cell (D, arrow) stained with Hematoxylin-Shorr and found in amniotic fluid from Nelore calves at parturition from Groups 1 (IVP), 2 (ET), and 3 (AI). (magnification = 1000x).

Table 1. Percentages of small intermediate, large intermediate, nucleated superficial, and anuclear superficial cells found in amniotic fluid from Nelore calves at parturition from Groups 1 (IVP), 2 (ET), and 3 (AI).

| Cell Type (%) | Group 1 (IVP) | Group 2 (ET) | Group 3 (AI) |
|---------------|-----------------------------------|-----------------------------------|-----------------------------------|
| SIC | 1.80 ^a (72/4000) | 2.98 ^b (119/4000) | 2.97 ^b (119/4000) |
| LIC | 7.10 ^a (283/4000) | 7.44 ^a (298/4000) | 6.82 ^a (273/4000) |
| NSC | 33.24 ^a (1330/4000) | 24.31 ^b (972/4000) | 30.65 ^c (1226/4000) |
| ASC | 57.86 ^a (2315/4000) | 65.27 ^b (2610/4000) | 59.56 ^a (2382/4000) |

Different superscript letters in the same row differ (Chi-square test; $P < 0.05$).

SIC: small intermediate cell, LIC: large intermediate cell, NSC: nucleated superficial cell, and ASC: anuclear superficial cell.

Regarding the immature cell types identified, the percentage of SIC from Group 1 was significantly lower ($P < 0.05$) when compared to Groups 2 and 3; however, there was no difference in the percentage of

LIC. There was a significant difference in the percentage of SNC for mature cell types among the 3 groups (Group 1 - highest percentage; Group 2 - lowest percentage). In relation to the percentage of ASC, Group 2 had a higher

percentage when compared to Groups 1 and 3.

The fetal maturity evaluation was determined by counting the keratinized cells, which is represented

by the percentage of these cells in the amniotic fluid as shown in Fig. 2. No difference among groups was observed for the KC cells ($P > 0.05$).

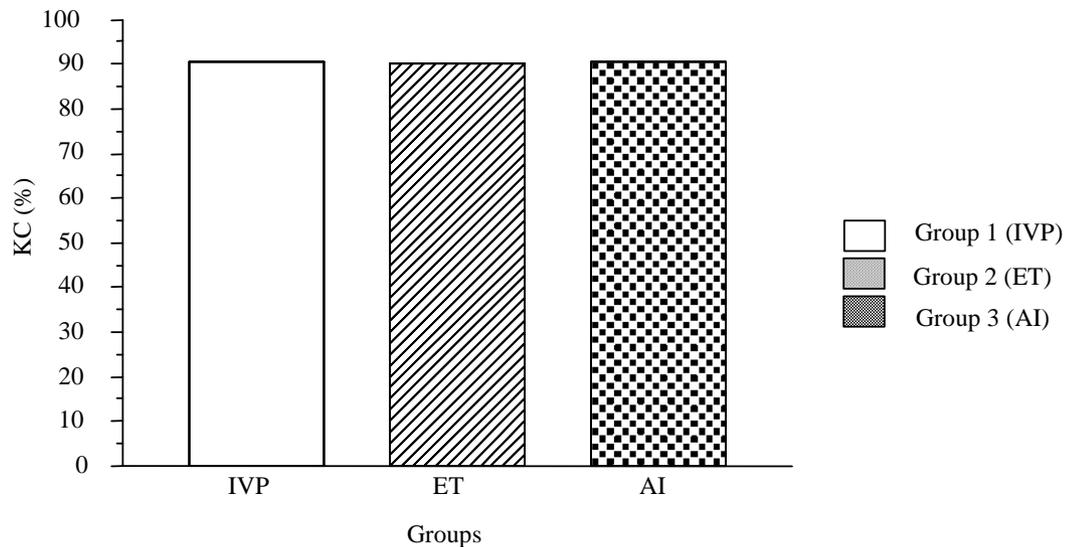


Figure 2. Keratinized cell percentages of cells stained with Hematoxylin-Shorr and found in amniotic fluid from Nelore calves at parturition from Groups 1 (IVP), 2 (ET), and 3 (AI).

Discussion

Amniotic fluid cytology analysis using Hematoxylin-Shorr is a safe and practical method for fetal evaluation in late gestation in humans, which offered the basis for the use of this staining technique in the current study. The amniotic fluid cytology staining is used in human medicine to characterize fetal epidermal maturity in late gestation (Agorastos 1979).

Determination of fetal maturity through cytology of the amniotic fluid is based on its morphologic variations or changes in its staining property (Cunha *et al.*, 1978). The Hematoxylin-Shorr was used in this study to qualify and quantify the cell types in the amniotic fluid and to evaluate the fetal maturity in bovine at the moment of parturition. Bongso and Basrur (1975) described that the cell's identity is not dependent on gestation stage when based on morphological characteristics, allowing a direct correlation between cell numbers and phase of gestation. The present study shows a small amount of cells in the amniotic fluid, despite that the samples were collected at parturition.

The authors of the current experiment, Barreto (2002), and Martins and Prestes (2003) classified the amniotic fluid cells in four types: I - immature or deep cells; II - mature or intermediate cells; III - mature cornified cells and nucleated or superficial cells; or IV - hyper-mature cornified and anuclear or squamous cells. In the current experiment, parabasal (deep) cells were not observed; however, the description of the remaining cells is similar to the cells described by the authors.

The percentage of superficial and anuclear cells

greater than 90% was found for fetuses at a post-mature stage (Martins and Prestes, 2003). No statistical differences were found among the three groups evaluated in respect to the percentages of keratinized cells ($90.0 \pm 5.0\%$), suggesting an epidermal mature stage although no difference in epidermal development among calves conceived from IVP, ET, or AI was observed. A post-maturity stage cannot be defined as previously described by Martins and Prestes (2003) since the calves conceived by AI (control) were not statistically different when compared to the remaining groups.

The amniotic fluid is an important source for fetal evaluation through analysis of cytology. It facilitates determining the degree of maturity of epidermal fetal cells with the use of Hematoxylin-Shorr staining, despite that there was no difference in epidermal development among calves of three groups from the current experiment.

Acknowledgments

Financial Support: FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo.

References

- Agorastos T. 1979. Antenatal estimation of fetal maturity by exfoliative cytology of amniotic fluid. *Z Geburtshilfe Perinatol*, 183:118-127.
- Arruda OB, Tolosa EML, Neto AGF. 1976. *Manual de técnicas para histologia normal e patológica*. São



- Paulo, SP: Editora da Universidade de São Paulo. 239pp.
- Barreto CS.** 2002. *Avaliação bioquímica, citológica e do perfil eletroforético de proteínas no líquido amniótico e alantoideano de cadelas entre 30 a 40 dias de gestação e no momento da cesariana.* Botucatu, SP, Brasil: Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista. Dissertation.
- Bongso TA, Basrur PK.** 1975. Prenatal diagnosis of sex in cattle by amniocentesis. *Vet Rec*, 96:124-126.
- Cunha SP, Valeri FV, Ferraz STA, Bailão LA, Carvalho RL.** 1978. Citologia do líquido amniótico corado pelo método de Harris-Shorr na gravidez acima da 40ª semana. *Rev Assoc Med Bras*, 24:243-245.
- Gartner LP, Hiatt JL.** 2003. Introdução à histologia e técnicas básicas de histologia. In: Tratado de histologia em cores. 2nd ed. Rio de Janeiro, RJ: Guanabara Koogan. pp.1-8.
- Goodhand KL, Watt RG, Staines ME, Hutchinson JSM, Broad-Bent PJ.** 1999. *In vivo* oocyte recovery and *in vitro* embryo production from bovine donors aspirated at different frequencies or following FSH treatment. *Theriogenology*, 51:951-961.
- Kjeldsberg C, Knight J.** 1993 Body fluids: laboratory examination of amniotic, cerebrospinal, serous and synovial fluids. In: American Society of Clinical Pathologist. 3rd ed. Chicago, USA: Theid. pp. 387.
- Leibfried-Rutledge ML.** 1999. Factors determining competence of *in vitro* produced cattle embryos. *Theriogenology*, 51:473-485.
- Malard PF, Cordeiro DM, Peixer MAS, Pereira DC, Marques Junior AP, Rumpf R.** 1999. Índice de recuperação, qualidade e potencial de desenvolvimento de ovócito de bezerros zebuínos de 2 a 4 meses de idade – resultados preliminares. *Arq Fac Vet UFRGS*, 27:256 (abstract).
- Martins LR, Prestes NC.** 2003. Ensaio sobre exame citológico do líquido amniótico de cadelas (*Canis familiaris*) colhido no momento da cesariana. *Ars Vet*, 19:294-299.
- Oliveira MLCS, Mota ARC, Viero RM.** 2000. *Citotecnologia: manual de normas técnicas.* Botucatu, SP: Laboratório de Citologia, Departamento de Patologia, Faculdade de Medicina. 24pp.
- Prestes NC.** 2005. Produção *in vitro* de embriões bovinos – problemas e desafios – Visão obstétrica. *Acta Sci Vet*, 33:119-124.
- Reichenbach HD, Oliveira MAL, Lima PF, Filho ASS.** 2002. Transferência e criopreservação de embrião bovinos. In: Golçalves PBD, Figueiredo, JR, Freitas VJF (Ed.). *Biotécnicas aplicadas à reprodução animal.* São Paulo, SP: Varela. pp.127-177.
- Schrage R, Bogelspacher HR, Wurster KG.** 1982. Amniotic fluid cells in the second trimester of pregnancy. *Acta Cytol*, 26:407-416.
- Taneja M, Bols PEJ, Velde V, Schreiber D, Tripp MV, Levine H, Echelard Y, Riesen J, Yang X.** 2000. Development competence of juvenile calf oocytes *in vitro* and *in vivo*: influence of donor animal, variation and repeated gonadotropin stimulation. *Biol Reprod*, 62:206-213.
- Tervit HR.** 1996. Laparoscopy oocytes recovery and juvenile breeding. *Anim Reprod Sci*, 42:227-238.
- Wagtendonk-Deleeuw AE, Mullaart AP, Roos W.** 2000. Effects of different reproduction techniques: AI, MOET or IVP, on health and welfare of bovine offspring. *Theriogenology*, 53:575-597.
-