

Induction of the acrosome reaction test to *in vitro* estimate embryo production in Nelore cattle

[*Teste de indução da reação acrossomal na estimativa de produção in vitro de embriões Nelore*]

M.Z. Costa, L.Z. Oliveira, M.V. Resende, A.C. Lucio, A.P. Perini,
M.C.V. Miguel, V.F.M.H. Lima*

Faculdade de Ciências Agrárias e Veterinárias - UNESP
Via de Acesso Prof. Paulo Donato Castellane s/n
14884-900 – Jaboticabal, SP

ABSTRACT

The effectiveness of induction of the acrosome reaction (AR) test as a parameter to *in vitro* estimate embryo production (IVP) in Nelore breed and the AR pattern by the Trypan Blue/Giemsa (TB) stain were evaluated. Frozen semen samples from ten Nelore bulls were submitted to AR induction and were also evaluated for cleavage and blastocyst rates. The treatments utilized for AR induction were: control (TALP medium), TH (TALP medium + 10µg heparin), TL (TALP medium + 100µg lysophosphatidylcholine) and THL (TALP medium + 10µg heparin + 100µg lysophosphatidylcholine). Sperm acrosomal status and viability were evaluated by TB staining at 0 and after 4h incubation at 38°C. The results obtained for AR presented a significant difference ($P<0.05$) in the percentage of acrosome reacted live sperm after 4h of incubation in the treatments that received heparin. The cleavage and blastocyst rates were 60% and 38% respectively and a significant difference was observed among bulls ($P<0.05$). It was founded a satisfactory model to estimate the cleavage and blastocyst rates by AR induction test. Therefore, it can be concluded that the induction of the AR test is a valuable tool to predict the IVP in Nelore breed.

Keywords: cattle, fertility, semen, acrosome reaction, fertilization

RESUMO

Avaliou-se a eficiência da técnica de indução da reação acrossomal (RA) como parâmetro para estimar a produção *in vitro* (PIV) de embriões Nelore e analisou-se o padrão de RA pela técnica de coloração Azul de Tripán/Giemsa (TB). Amostras de sêmen congelado de dez touros foram submetidas à indução da RA e avaliadas quanto a taxa de clivagem e blastocisto. Os tratamentos utilizados para indução da RA foram: controle (meio TALP), TH (meio TALP + 10µg heparina), TL (meio TALP + 100µg lisofosfatidilcolina) e THL (meio TALP + 10µg heparina + 100µg lisofosfatidilcolina). Avaliou-se viabilidade espermática e acrossomal pela coloração TB a zero e após 4h de incubação a 38°C. Os resultados obtidos para RA mostram uma diferença significativa ($P<0,05$) na porcentagem de espermatozoides vivos com acrossoma reagindo após 4h de incubação nos tratamentos que receberam heparina. As taxas de clivagem e blastocisto obtidas foram 60% e 38% respectivamente e observou-se uma diferença significativa entre touros ($P<0,05$). Delineou-se um modelo satisfatório para estimar as taxas de clivagem e blastocisto. Desta forma, conclui-se que o teste de indução da RA é uma ferramenta valiosa para prever a PIV na raça Nelore.

Palavras-chave: bovino, fertilidade, sêmen, reação acrossomal, fertilização

Recebido em 9 de março de 2009

Aceito em 16 de julho de 2010

*Autor para correspondência (*corresponding author*)

E-mail: veralima@fcav.unesp.br

INTRODUCTION

It is well established that the sire can have a major effect on the outcome of *in vitro* fertilization (IVF) (Hillery et al., 1990) and in terms of cleavage rates and blastocyst yield among semen samples from different bulls (Ward et al., 2001; 2003). Those parameters can be used as important indicators for *in vitro* bull fertility. In the animal improvement programs which utilize *in vitro* production of embryos (IVP) as a genetic multiplication tool, the utilization of a predictive method for cleavage rates and IVP of a particular bull semen samples is important for saving laboratory time and unnecessary procedural costs.

When frozen semen from bulls with high field fertility was compared with that from bulls of low field fertility, a significant difference between the two groups was found in both cleavage and blastocyst yield (Zhang et al., 1997).

The ability in predicting the fertility of semen with laboratory tests is still limited, mainly due the complexity of the sperm and the fertilization process. Therefore, it is necessary to enhance the prediction of bull fertility with other tests in order to increase the accuracy of their reproductive potential estimative.

One of the key processes in mammalian fertilization is the acrosome reaction (AR) usually triggered in spermatozoa upon their binding to the zona pellucida of the egg. The AR involves fusion between the plasma membrane and the underlying outer acrosomal membrane, as a result of which the acrosomal contents are released.

The AR can be induced by either natural inducers as zona pellucida (Florman, 1994) and progesterone (Oehninger et al., 1994) or artificial inducers as heparin (Parrish et al., 1988; Kitiyanant et al., 2002), lysophosphatidylcholine (Parrish et al., 1988) and calcium ionophore (Christensen et al., 1996; Kitiyanant et al., 2002).

Utilization of the *in vitro* induction of the AR test as described by Januskauskas et al. (2000a,b) and greater implementation of IVF techniques have stimulated interest in evaluating the

fertilizing potential of Nelore semen using this particular *in vitro* combination.

The IVF employment with this aim has been widely studied being already established in the literature the importance of this technique, besides the high costs involved. As well, significant correlations are described between AR responsiveness and fertility (Januskauskas et al., 2000a,b; Stahr et al., 2000).

Nowadays, several methods have been used as an attempt to predict the potential fertility of semen. One is trying to estimate the potential fertility of a semen sample, with has not been successfully done to date. Conventional viability tests included sperm morphology, motility, acrosome integrity, and abnormal DNA condensation. Methods for evaluation of the sperm plasmalemma included eosin/nigrosin (EN) and trypan-blue vital stains, propidium iodide (PI) in combination with carboxyfluorescein diacetate (CFDA) or SYBR-14 (SYBR) fluorescent vital stains, and the hypoosmotic swelling test (HOST) (Brito et al., 2003). Innovations in stains techniques have offered new means of spermatozoa functionality evaluation in several species (Garner and Johnson, 1995; Arruda et al., 2002; Celeghini et al., 2005). However, some costly equipment is necessary.

The Trypan-Blue/Giemsa stain (TB) was used to evaluate the efficiency of the sperm AR induction in estimating IVP from oocytes of Nelore cattle. This staining method of sperm evaluation is easy, less expensive, and efficient and could be used as a test for predicting the potential fertility of bovine (Kitiyanant et al., 2002; Tartaglione and Ritta, 2004) and equine semen samples (Kutvolgyi et al., 2006). Still in relation to TB staining, Kovacs and Foote (1992) also stated that simultaneous evaluation of the viability and acrosome integrity of sperm allows differentiation of true acrosome reaction from degenerative acrosome loss after cell death.

The aim of the present work was to evaluate, in Nelore breed bulls, the efficiency of the induction of the AR technique as a parameter to estimate the IVP and the acrosomal reaction pattern by TB stain.

MATERIAL AND METHODS

Frozen semen samples of ten Nelore bulls were evaluated. Straws from six different batches (six different ejaculates) of each bull were thawed by immersion in water-bath at 35°C for 30sec and homogenized. The sperm were evaluated to sperm progressive motility and vigor. In order to remove the extender, the samples were washed three times in TALP medium with BSA-V (Sigma A-2153) under centrifugation (250 x g during 10min). After the last wash, the sediment was suspended in 4mL of TALP medium and the motility and vigor were evaluated again. Afterward, the semen concentration was determined from a 20µL sample in Neubauer Count and adjusted to 20x10⁶ sperm/mL.

The semen samples were submitted to different treatments: control (TALP medium), TH (TALP medium + 10µg heparin), TL (TALP medium + 100µg lysophosphatidylcholine; LPC) and THL (TALP medium + 10µg heparin + 100µg LPC). The heparin (Sigma H-9399) in the final concentration of 10µg/mL was utilized to induce the *in vitro* sperm capacitation and the LPC (Sigma L-5004) in the final concentration of 100µg/mL was utilized in order to induce the acrosomal reaction. Both were prepared according to Parrish et al. (1988). In the beginning of incubation (0h), the LPC was added in tubes (5µg/microtube) and after 4h of incubation, it was added to other four tubes. At each hour of reaction sperm were microscopically evaluated on heated slides (~37.5°C) under a coverslip for motility and vigor. The tubes with one of the four treatments were incubated in water-bath at 38°C for 4h and manually agitated at each 15 to 20min.

Acrosome reaction evaluations were carried out using 10µL-semen smears that had been stained with Trypan-Blue/Giemsa (TB) according to Kovacs and Foote (1992). In each smear layer, 200 spermatozoa were analyzed and eight distinct spermatozoal classes were identified: 1) intact alive (IA); 2) damaged alive (DA); 3) alive with detached acrosome (SA); 4) alive with acrosome reaction (RA); 5) intact dead (ID); 6) damaged dead (DD); 7) dead with detached acrosome (SD); and 8) dead with degenerate acrosome (RD).

Cumulus oocytes complexes (COC) were obtained by aspiration of 2-8mm follicles from the ovaries of slaughtered cows up to 3h after slaughter. The COC were selected and transferred into microdroplets of TCM-199 medium (GIBCO BRL, Grand Island, USA) supplemented with 10% bovine fetal serum; 26.2mM NaHCO₃; 0.2mM of sodium pyruvate; 75µg Kanamicina/mL; 0.5µg FSH/mL; 100µg eCG/mL, and 1µg estradiol/mL (maturation medium) and cultivated for 24h at 38.5°C in atmosphere of 5% of CO₂ in air and relative humidity of 100%. The semen straws were thawed at 35°C for 20sec and the semen was deposited on a discontinuous Percoll® (Amersham Pharmacia Biotech AB, Uppsala, Sweden) density gradient. To prepare the gradient, 2.0mL of 45% (v/v) Percoll, diluted with HEPES (10mM) buffered TALP, was placed over 2.0mL 90% (v/v) Percoll for 30min at 700 x g at 22°C. Viable sperm from the pellet had concentration adjusted to 25x10³ sperm/mL and 4µL of those were added to each microdroplets (100µL) with IVF medium (TALP medium supplemented with 2.7µg penicillin, 10µg hypotaurine, 0.33µg epinephrine, and 10UI heparin/mL). The final sperm concentration in the fertilization microdroplets was 100x10³ sperm and they remained incubated for 60 to 90min for sperm capacitation. The oocytes were matured for 24 to 25h and washed three times in TCM-199 culture medium (GIBCO BRL) supplemented with 25mM HEPES, 0.2mM sodium pyruvate, and 0.3% BSA (10mg/mL; fraction V, fatty acid free - Inlab, Sao Paulo, Brazil) and once in microdroplet of IVF medium. The oocytes and sperm (approximately 25 oocytes per microdroplet of 100µL) were incubated for 10 to 24h at 38.5 to 39°C in 5% of CO₂ in air. After fertilization, the presumed zygotes were co-cultivated with granulose cells, performed in a humidified atmosphere of 5% CO₂, in air, 100% of relative humidity and at temperature of 38.5 to 39°C. After 33h of culture, the cleavage rate was evaluated and embryos were cultivated replacing the medium at each 48h until the seventh day when blastocysts rate was evaluated. Three repetitions of the experiment were performed in different days for each bull (with different semen batches for each repetition) and 40 oocytes were utilized per repetition. The order of utilization of the bull semen and batches were randomly in order to

avoid a possible IVF effect (day/oocyte) in the semen evaluation.

The variance analysis of the acrosomal reaction and IVP data was performed by Tukey's Test. The inter-relations between the acrosomal reaction and IVF variable were determined by correlation analysis (Pearson's) and linear regression study (stepwise regression analysis). Data obtained from the experimental procedure were analyzed using Statistical Analysis System software (SAS Institute - Cary, NC, USA), 6.12 version. All statistical analyses were calculated with a significance level of 5% (differences were considered significant at $P < 0.05$).

RESULTS

The *in vitro* AR induction was analyzed by light microscopy utilizing TB stain (Kovacs and Foote, 1992). The analyses of the different treatments demonstrate a significant difference ($P < 0.01$) to the percentage of the RA sperm between the THL treatment (43%) and the other treatments (TALP:36%, TH:39%, and TL:36%) at hour zero. After 4h of incubation, a significant difference ($P < 0.01$) could be observed in the percentage of the reacted alive sperm to the treatments which received heparin (TH:56% and THL:57%) in relation to the others (TALP:52% and TL:53%). The heparin treatment (TH) proved to be an important inductor of acrosomal reaction in relation to the control treatment ($P < 0.01$) after 4h of incubation and the increase in the percentage of reacted alive spermatozoa was 5.5%. The LPC treatment (TL) was not significantly different from control treatment neither 0 nor at 4h in relation to the proportion of reacted alive spermatozoa. Also, no significant difference was observed from the results of treatments with heparin and LPC (THL) in relation to heparin treatment (TH) after incubation for 4h. LPC did not prove to have the expected inductor effect, presenting similar

results to the control treatment (TL=TALP), or to the treatment with heparin (THL=TH). The different treatments did not express any negative effect on the sperm viability during the 4h of incubation. There were no variations in the average of the dead sperm (13%) for the different treatments.

The means of the cleavage and blastocyst rates were compared by Turkey test at significance level of 5%. It was observed a difference ($P < 0.05$) in the cleavage and blastocyst rates among bulls (Table 1).

Table 1. Means of cleavage and blastocyst rates in percentages for *in vitro* produced bovine embryos that had been fertilized using thawed, cryopreserved sperm from ten Nelore bulls

Bull	Means of cleavage rates	Means of blastocyst rates
1	52.1bcd	32.6bc
2	68.6ab	49.1a
3	65.8abc	42.2ab
4	65.4abc	46.6ab
5	43.6d	36.3abc
6	68.7ab	36.5abc
7	50.9cd	32.5bc
8	61.0abc	38.2abc
9	71.1a	41.7ab
10	61.4abc	25.7c
Average	60.8	38.1

Means followed by different superscripts (a,b,c,d) within the same column differ significantly ($p < 0.05$).

The correlations obtained among thawed, cryopreserved semen samples from ten Nelore bulls in the different sperm classes (for each treatment at 0 and 4h incubation) and cleavage and blastocyst rates are described in Tables 2 and 3, respectively. Highly negative correlations were observed between the acrosomal intact alive classes for the different sperm treatments and embryonic cleavage rates (Table 2) as well as the acrosomal intact alive classes and the blastocyst development rates (Table 3).

Table 2. Correlation coefficients (r values) between the only significant classification of sperm function, i.e. intact alive sperm (IA) and cleavage rates of *in vitro* produced embryos for different media treatments (a) at 0 and 4h incubation

	TALP 0h	TALP 4h	TH 0h	TH 4h	TL 0h	TL 4h	THL 0h	THL 4h
IA	-0,76*	-0,75*	-0,81**	Ns	-0,80*	-0,67*	-0,73*	Ns

* $P < 0.05$; ** $P < 0.01$; ns: not significant.

(a): TALP (TALP medium), TH (TALP medium + 10 μ g heparin), TL (TALP medium + 100 μ g LPC), THL (TALP medium + 10 μ g heparin + 100 μ g LPC)

Induction of the acrosome reaction...

Table 3. Correlation coefficients (r values) between certain sperm class variables (a) and the developmental rates of blastocysts *in vitro* produced using different treatments (b) after 0 and 4h incubation

	TALP 0h	TALP 4h	TH 0h	TH 4h	TL 0h	TL 4h	THL 0h	THL 4h
IA	-0,740*	-0,811**	-0,719*	Ns	-0,669*	-0,745*	ns	Ns
RA	Ns	0,645*	Ns	Ns	ns	ns	ns	Ns

*P<0.05; **P<0.01; ns: no significant.

(a): IA (intact alive sperm), RA (acrosome-reacted alive sperm);

(b): TALP (TALP medium), TH (TALP medium + 10µg heparin), TL (TALP medium + 100µg LPC), THL (TALP medium + 10µg heparin + 100µg LPC).

The regression study between induction of the AR and cleavage found a mathematical model (cleavage = 73.5824 – 0.3553 x IA TALP 4h; **R²:65.93%) of simple linear regression that allows to predict the cleavage results utilizing the induction of the AR technique with 65% of reliability. The regression study between induction of the AR and blastocyst found a mathematical model (blastocyst = 16.2339 + 0.2777 x RA TH 0h + 2.4577 x RA TH 4h/RA TH 0h + 0.6033 x RD THL 0h – 0.0972 x RA THL 4h – RA THL 0h; **R²:99.52%) of multiple linear regression that allows to predict the blastocyst results by the induction of the AR technique with 99% of truthfulness.

DISCUSSION AND CONCLUSION

The present work proposes the utilization of induction of the AR, evaluated by TB stain, as an alternative in the prediction of the IVP potential in the Nelore breed. The stain technique was efficient not only in the spermatic viability detection but also in the detection of acrosoma presence or absence according to similar results described by Kovacs and Foote (1992).

The heparin confirmed to be an important inductor of acrosomal reaction after 4h of incubation in sperm from *Bos indicus* sires. This finding is according to Parrish et al. (1988) that described that heparin as one of the most efficient agents in the sperm capacitation induction after a minimal exposure of 4h in taurine sires. Although the literature describes an important participation of LPC in acrosomal reaction of the *in vitro* capacitated sperm (Parrish et al., 1988), the results of this work indicate that LPC did not present the expected inductor effect in the Nelore semen. Therefore, it became clear the necessity of performing other works utilizing LPC as a RAI inductor agent for the *Bos indicus* in order to detect possible differences

between the bovine subspecies in relation to the concentration and methodology of the inductor agent preparation.

This study demonstrated a significant difference in cleavage and blastocyst rates among bulls (Table 1). These findings are in agreement with Ward et al. (2003) who also observed a significant variation *in vivo* among bulls in those parameters. It can be concluded, that the assessment of the potential fertility of the semen is necessary before performing the IVP. Moreover, according to studies from Januskauskas et al. (2000a,b) with *Bos taurus* and Stahr et al. (2000) with boars, the present study with *Bos Taurus indicus* demonstrated a high negative association between the intact alive (IA) sperm class and the cleavage and blastocysts rates. Hence, it can be indicated the importance of this sperm class in the prediction of those fertility parameters.

The negative correlations between IA class and cleavage rate verified in Table 2 indicates that an increase in the percentage of IA sperm cause a reduction in cleavage rate. Similar results were obtained in the correlation between the IA class and blastocyst rates (Table 3), also expressing this negative correlation in relation to this fertility parameter. It could also be verified a positive correlation between the RA class and blastocyst rate after 4h of incubation. A possible explanation for the negative correlations between the IA sperm at zero hour of incubation and the fertility rates evaluated, suggests the participation of live sperm which undergo spontaneous acrosome reaction in the fertilization process, as described by Tulsiani et al. (1998). The high negative correlation observed between IA class and cleavage and blastocyst rates confirms its importance in the prediction of bull fertility.

Lonergan (1994) described that blastocyst rate demonstrated to be more important than cleavage rate in the estimation of *in vitro* bull fertility. However, the difference in cleavage and blastocyst rates among bulls demonstrated in this study, indicates that the former is a model of better sensibility ($P < 0.05$) in relation to blastocyst rate ($P < 0.10$) in the detection of probable differences among semen samples of bulls. In addition, Zhang et al. (1997) described a significant positive correlation between *in vitro* cleavage rate and *in vivo* fertility, expressed by the non-return rates after AI.

Amann et al. (1993), Brito et al. (2003), and Gadea (2005) pointed out that the combination of tests to evaluate sperm function is the most reliable approach to increase the accuracy of estimating the potential fertility of semen samples. Rodriguez-Martinez and Barth (2007) also stated that a laboratory test that accurately estimates the potential fertility of a semen sample or a sire is distant and due to the complex nature of male fertility any sought for laboratory method must include testing of most sperm attributes relevant for both fertilization and embryo development.

Nevertheless, the statistical associations (correlation and regression studies) found in this work suggest that AR induction is a prospective technique in the prediction of IVP results for semen from Nelore bulls. The main advantage of this technique is the facility of execution and lower costs. Therefore, the increasing efficiency and costs reduction in bull selection by utilization of this technique can be a useful tool in the increment of the animal improvement.

ACKNOWLEDGMENTS

The authors acknowledge FAPESP for the funding supplies 00/05348-5, 04/06044-0, and 05/59357-9.

REFERENCES

ARRUDA, R.P.; SOUZA, N.L.; MARQUES, A. et al. Evaluation of techniques using CFDA/PI, H258/FITCPSA and trypan blue/giemsa for assessment of the viability and acrosomal integrity of cryopreserved equine spermatozoa. *Theriogenology*, v.57, p.477, 2002.

AMANN, R.P.; HAMMERSTEDT, H.P. *In vitro* evaluation of sperm quality: an opinion. *J. Androl.*, v.14, p.397-405, 1993.

BRITO, L.F.C.; BARTHA, A.D.; BILODEAU-GOESELSB, S. et al. Comparison of methods to evaluate the plasmalemma of bovine sperm and their relationship with *in vitro* fertilization rate. *Theriogenology*, v.60, p.1539-1551, 2003.

CELEGHINI, E.C.C.; NASCIMENTO, J.; ANDRADE, A.F.C. et al. Use of CMXRos and JC-1 on mitochondrial function evaluation, associated to fluorescent probes to plasmatic and acrosomal membranes evaluation in bovine spermatozoa. *Acta Sci. Vet.*, v.33, p.321 (abstract), 2005.

CHRISTENSEN, P.; WHITFIELD, C.H.; PARKINSON, T.J. *In vitro* induction of acrosome reactions in stallion spermatozoa by heparin and A23187. *Theriogenology*, v.45, p.1201-1210, 1996.

FLORMAN, H.M. Sequential focal and global elevations of sperm intracellular Ca^{2+} are initiated by the zona pellucida during acrosomal exocytosis. *Dev. Biol.*, v.165, p.152-164, 1994.

GADEA, J. Sperm factors related to *in vitro* and *in vivo* porcine fertility. *Theriogenology*, v.63, p.431-444, 2005.

GARNER, D.L.; JOHNSON, L.A. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biol. Reprod.*, v.53, p.276-284, 1995.

HILLERY, F.L.; PARRISH, J.J.; FIRST, N.L. Bull specific effect on fertilization and embryonic development *in vitro*. *Theriogenology*, v.33, p.249 (abstract), 1990.

JANUSKAUSKAS, A.; JOHANNISSON, A.; SODERQUIST, L. et al. Assessment of the fertilizing potential of frozen-thawed bovine sperm by calcium ionophore A23187-induced acrosome reaction *in vitro*. *Theriogenology*, v.53, p.859-875, 2000a.

JANUSKAUSKAS, A.; GIL, J.; SODERQUIST, L. et al. Relationship between sperm response to glycosaminoglycans *in vitro* and non return rates of Swedish dairy AI bulls. *Reprod. Domest. Anim.*, v.35, p.207-212, 2000b.

Induction of the acrosome reaction...

- KITIYANANT, Y.; CHAISALEE, B.; PAVASUTHIPAISIT, K. Evaluation of the acrosome reaction and viability in buffalo sperm using two staining methods: the effects of heparin and calcium ionophore A23187. *Int. J. Androl.*, v.25, p.215-222, 2002.
- KOVACS, A.; FOOTE, R.H., Viability and acrosome staining of bull, boar and rabbit sperm. *Biotech. Histochem.*, v.67, p.119-124, 1992.
- KUTVOLGYI, G.; STEFLER, J.; KOVACS, A. Viability and acrosome staining of stallion sperm by Chicago sky blue and Giemsa. *Biotech Histochem.*, v.81, p.109-117, 2006.
- LONERGAN, P. The application of *in vitro* fertilization techniques to the prediction of bull fertility. *Reprod. Domest. Anim.*, v.29, p.12-21, 1994.
- OEHNINGER, S.; BLACKMORE, P.; MORSHEDI, M. et al. Defective calcium influx and acrosome reaction (spontaneous and progesterone-induced) in spermatozoa of infertile men with severe teratozoospermia. *Fertil. Steril.*, v.61, p.349-354, 1994.
- PARRISH, J.J.; SUSKO-PARRISH, J.; WINER, M.A. et al. Capacitation of bovine sperm by heparin. *Biol. Reprod.*, v.38, p.1171-1180, 1988.
- RODRIGUEZ-MARTINEZ, H.; BARTH, A.D. *In vitro* evaluation of sperm quality related to *in vivo* function and fertility. *Soc. Reprod. Fertil.*, v.64, Suppl., p.39-54, 2007.
- STAHR, B.; BERG, C.; BECHTOLD, B. et al. Relations between the amount of *in-vitro*-capacitated sperm cells after liquid-preservation and the fertility of AI-boars. In: INTERNATIONAL MEETINGS ON ANIMAL REPRODUCTION, 14., 2000, Stockholm. *Proceedings...* Stockholm: ICAR. v.2, p.152, 2000.
- TARTAGLIONE, C.M.; RITTA, M.N. Prognostic value of spermatological parameters as predictors of *in vitro* fertility of frozen-thawed bull semen. *Theriogenology*, v.62, p.1245-1252, 2004.
- TULSIANI, D.R.P.; ABOU-HAILA, A.; LOESE, C.R. et al. The biological and functional significance of the sperm acrosome and acrosomal enzymes in mammalian fertilization. *Exp. Cell Res.*, v.240, p.151-164, 1998.
- WARD, F.; RIZOS, D.; CORRIDAN, D. et al. Paternal influence on the time of first embryonic cleavage post insemination and the implications for subsequent bovine embryo development *in vitro* and fertility *in vivo*. *Mol. Reprod. Dev.*, v.60, p.47-55, 2001.
- WARD, F.; RIZOS, D.; BOLAND, M.P. et al. Effect of reducing sperm concentration during IVF on the ability to distinguish between bulls of high and low field fertility: work in progress. *Theriogenology*, v.59, p.1575-1584, 2003.
- ZHANG, B.R.; LARSSON, B.; LUNDEHEIM, N. et al. Relationship between embryo development *in vitro* and 56-day non return rates of cows inseminated with frozen-thawed semen from dairy bulls. *Theriogenology*, v.48, p.221-231, 1997.