Relation between Bulls and Semen Preparation on In Vitro Produced Bovine Embryos

Clara Larocca[1], Juan E. Romano[2], Juan Calvo[3], Ignacio Lago[1], Danilo Fila[1], Gonzalo Roses[1], Monica Viqueira[3], Sergio Kmaid[1] and Kei Imai[4]*

[1]Laboratorio de Transplante de Embriones y Biotecnicas, [2]Departamento de Fisiologia, [3]Departamento de Morfologia y Desarrollo, Facultad de Veterinaria, Lasplaces 1550, 11600 Montevideo, Uruguay and
[4]National Livestock Breeding Center, Ministry of Agriculture, Forestry and Fisheries, Nishishirakawa-gun, Fukushima 961, Japan

Abstract: The objective of this experiment was to compare the effectiveness of two bulls and two different semen treatments on rates of in-vitro fertilization of in-vitro matured bovine oocytes, cleavage and development of embryos to the stage of morula and blastocyst by using a 2 × 2 factorial design. Oocytes (n=946) aspirated from follicles 2–6 mm in diameter were incubated for 20–22 h in TCM-199 supplemented with 5% calf serum, and then inseminated with frozen-thawed semen from two single ejaculates of two Holstein bulls of proven fertility. For the conventional washing method (C), semen was washed twice by centrifugation at 500 g for 5 min with modified BO medium (BO: without glucose or bovine serum albumin (BSA)) supplemented with 10 mM caffeine and 2.5 IU/ml heparin (BO-wash), and then diluted with the same amount of BO containing 20 mg/ml BSA (BO-dilute). For the Percoll washing method (P) semen was layered on a 45 to 90% percoll gradient in a 15 ml centrifugation tube. After 30 min of centrifugation at 700 g, the sperm pellet was recovered from the bottom of the tube, and then resuspended in BO-wash and double diluted with BO-dilute. With this sperm suspension insemination drops (100 μl) were prepared and the sperm was cultured with oocytes for 5 h. Then the oocytes were transferred to CR1aa supplemented with 5% calf serum for 7 days. The rates of cleavage and morula or blastocyst development of oocytes fertilized in vitro were similar in P (14.2% and 7.9%, respectively) and C (17.7% and 7.7%, respectively) methods for bull A, but these rates were significantly higher (P<0.005) with C (72.0% and 46.9%, respectively) method than with P (58.7% and 28.2%, respectively) method for bull B. In total rates of fertilization, cleavage and morula or blastocyst (combined data for C and P methods) were significantly higher (P<0.05) for bull B (83.8%, 65.8% and 33.9%, respectively) than those for bull A (42.4%, 16.0% and 7.9%, respectively). These results indicate that the Percoll washing method is not superior to the conventional washing method for in-vitro production of bovine embryos independently of the significant differences between bulls.

Key words: Bovine, Semen, Percoll separation, In vitro fertilization, In vitro development.

One of the important factors for successful fertilization *in vitro* in mammals is the proper induction of capacitation and acrosome reaction of sperm. Differences among bulls in the ability of sperm to fertilize oocytes were reported several times with the conventional IVF method [1–3] and considered to be an obstacle to the worldwide use of this technology [4], and also it was reported that many factors may interact to produce non repeatable results with the conventional IVF system [1, 3, 5]. To solve this problem the use of heparin was reported as a substance which collaborates to stimulate capacitation of sperm [6, 7]. Most recently Parrish *et al.*, [8] have recommended Percoll washing for preparation of sperm for use in routine bovine IVF with frozen-thawed semen, because they found that a high percentage of motile sperm can be obtained with Percoll washing without decreasing the resultant morulae and blastocysts. Nevertheless, insufficient information ex-

Received: November 11, 1996
Accepted: February 26, 1997
*To whom correspondence should be addressed.

ists on which procedures are most effective in making frozen-thawed bull spermatozoa capable of being capacitated and able to fertilize IVM oocytes resulting in high rates of *in-vitro* production (IVP) of embryos.

The objective of the present experiment was to investigate the variation in frozen-thawed bull spermatozoa treated by conventional and Percoll washing methods and the effects of the sperm treatments on the ability of sperm to fertilize IVM oocytes and IVP of embryos.

Materials and Methods

Collection and in vitro culture of oocytes

Ovaries were obtained from a local slaughterhouse and transported to the laboratory at 30–35°C in physiological solution (0.9%) with 100 μg/ml Gentamycine (Gentamycine, Laboratories Herix S.A., Montevideo, Uruguay) within 3 h of collection. Surface follicles (2-6 mm in diameter) were punctured with an 18 G hypodermic needle connected to a 5 ml syringe. Only healthy-looking oocytes with compact cumulus cells were selected and washed 3 times with modified PBS (m-PBS: Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 1 mg/ml glucose, 36 mg/l sodium pyruvate, 5% calf serum (CS, Gibco BRL, Grand Island, NY, U.S.A.), 100 IU/ml penicillin (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO, U.S.A). And then oocytes (70 to 100) were cultured for 20–22 h in a drop of 600 μl 25 mM Hepes buffered TCM-199 (199, Gibco BRL, Grand Island, NY, U.S.A) containing 5% CS covered with paraffin oil (Mineral oil, E.R. Squibb & Sons, Inc. Princeton, NJ, U.S.A.). All incubations were conducted at 38.5°C in 99% relative humidity and 5% CO_2 in air.

Sperm preparation

Frozen semen from two Holstein bulls of proven fertility were used for the experiments. For each replicate and semen preparation procedure, 3 straws (0.5 ml) from each bull were thawed in water at 35°C for 30 sec. All doses of semen used in the three replicates were from one ejaculate of each bull.

Conventional sperm washing (C) method

For the first procedure the thawed semen were washed twice (centrifugation at 500 g for 5 min with modified Brackett and Oliphant medium (BO) [9] without glucose or bovine serum albumin (BSA)). BO medium was supplemented with 10 mM caffeine (Caffeine sodium benzoate. Sigma Chemical, St. Louis, MO, U.S.A.) and 5 IU/ml heparin (Heparin. Sigma Chemical, St. Louis, MO, U.S.A.). The semen concentration was adjusted to 12.5×10^6 cells/ml with BO dilution medium containing 20 mg/ml of BSA (Crystallized and lyophilized. Sigma Chemical, St. Louis, MO, U.S.A.) without heparin and caffeine. One hundred μl insemination drops covered with paraffin oil were prepared with this sperm suspension, with a final concentration of 5 mM caffeine, 2.5 IU/ml heparin and 10 mg/ml BSA.

Percoll washing (P) method

Motile spermatozoa were isolated by centrifugation through a two-step Percoll gradient with 90% in the lower layer (2 ml) and 45% in the upper layer (2 ml) [8]. One ml aliquots of thawed semen were layered over the upper layer of the Percoll gradient which had previously been warmed in a water bath at 38°C, and centrifuged at 700 g for 30 min. After washing, the supernatant was eliminated and the pellet of semen was resuspended in the same BO medium as used for the C method. The sperm concentration of the suspension was adjusted to 12.5×10^6 cells/ml.

IVF of IVM oocytes and in-vitro culture of embryos

IVM oocytes were taken out of maturation drops, washed in BO medium containing 10 mg/ml BSA and transferred to fertilization drops (20 to 25 oocytes/a drop) with a minimum amount of medium and incubated for 5 h with sperm. After insemination, ova were washed twice in CR1aa-CS (CR1aa containing 5% CS) [10] and cultured for 168 h in a drop of 600 μl medium covered with paraffin oil. After 48 h and 168 h of insemination, rates of cleavage and the development of embryos to the stage of morula and blastocyst, respectively, were recorded.

Examination of fertilization

At 18 to 20 h after insemination, some oocytes were mounted, fixed (acetic acid: methanol = 1:3), stained with 0.5% acetic orcein and examined for evidence of fertilization. Oocytes were considered as fertilized when they had enlarged sperm heads or male pronucleus (ei) and their corresponding sperm tail(s) in the cytoplasm. Oocytes with more than two pronuclei and 1st and 2nd polar bodies but with no sperm tails were also considered penetrated, because after 18 to 20 h of insemination sperm tails become invisible.

Statistical analysis and experimental design

Experimental design was a 2×2 factorial comparing two bulls and two different procedures for semen treatments. Statistical comparisons were made using Chi-square test (χ^2-test) with Yates' correction.

Results

The fertilization rate (combined data for C and P methods) for bull B (83.8% : 31/37) was significantly higher (P<0.005) than that for bull A (42.4% : 14/33). As shown in Table 1, the rates of cleavage and morula or blastocyst for bull A were similar for C (17.7% and 7.7%, respectively) and P (14.2% and 7.9%, respectively) methods. In contrast, these results showed significantly higher rates (P<0.005) for bull B with C (72.0% and 46.9%, respectively) method than with P (58.7% and 28.2%, respectively) method. The total rates of cleavage and morula or blastocyst (combined data for C and P methods) for bull B (65.8% and 33.9%, respectively) were significantly higher (P<0.0001) than those for bull A (16.0% and 7.9%, respectively).

Discussion

Successful IVM-IVF in cattle has been reported [11–13], since the first calf was born from an IVM-IVF embryo in 1985 [14], but the conventional IVF procedure was subject to variability caused by both variability amongst bulls [2, 15–17] and by subtle differences in the condition of the semen [18–21] that was used. To obtain a high and repeatable *in-vitro* fertilization frequency, the establishment of sperm capacitation technique that can be effective for sperm from any bulls is urgently required. In other words, there are problems in achieving fertilization *in vitro* for many desirable bulls and this needs to be solved by determining universal sperm capacitation techniques [4]. For this purpose caffeine [11, 22] or heparin [6] was reported to enhance the ability of bovine sperm to fertilize IVM oocytes *in vitro*. And recently, to obtain sperm being able to penetrate oocytes, most studies on IVF in cattle have used swim-up separated sperm [7, 23], but the procedure is complicated. More recently, Parrish *et al.* [8], have reported that more motile sperm can be obtained by sperm separation by a simple Percoll gradient procedure than by the swim-up procedure and they found that development to the morula or blastocyst stage of embryos sired by swim-up or Percoll separated sperm was the same. Nevertheless, in the present study, the cleavage rate and rate of morula or blastocyst development of oocytes fertilized *in vitro* were significantly higher for bull B when sperm was simply washed according to the conventional washing method than by the Percoll washing method. Although we used BO solution supplemented with heparin and caffeine, but Parrish *et al.* [8] used TALP solution supplemented with heparin for IVF media. These different concentrations of heparin, caffeine or BSA in IVF media may have an effect on the viability of sperm. Furthermore, Parrish *et al.* [8] reported that the Percoll washing method resulted in less ova being penetrated and cleaved than did the swim-up method. In contrast bull A had similar rates of development of embryos with both the Percoll and conventional washing methods. This may be caused by the fact that the rates of fertilization and cleavage were too low with bull A to have the effect of washing spermatozoa on the development of embryos compared with bull B with high rates of fertilization and cleavage.

Niwa *et al.* [1], reported great variation in penetration rates among the different bulls. In the present results it was also shown that for bull B the fertilization rate was significantly higher than that for bull A. Because the Percoll washing method could not improve the fertilization rates for bull A, more experiments are needed to establish universal sperm capacitation techniques.

Although the number of bulls used in this experiment was small, it can be concluded that under our conditions the Percoll washing method is not superior to the conventional washing method for IVP of bovine embryos.

Table 1. Effects of different bulls and sperm washing methods on rates of IVM-IVF and IVP of embryos

Sperm washing method	Bull A (%)			Bull B (%)		
	No. of oocytes inseminated	No. of cleaved oocytes	No. of embryos developed*	No. of oocytes inseminated	No. of cleaved oocytes	No. of embryos developed
Conventional (C)	248 (3)**	44 (17.7)^{a}	19 (7.7)^{b}	239 (3)	172 (72.0)^{e}	112 (46.9)^{f}
Percoll (P)	253 (3)	36 (14.2)^{a}	20 (7.9)^{b}	206 (3)	121 (58.7)^{c}	58 (28.2)^{d}
Total	501	80 (16.0)^{g}	39 (7.9)^{i}	445	293 (65.8)^{h}	170 (33.9)^{i}

*No. of embryos developed to the stage of morula and blastocyst after 168 h of insemination.
**Replication.
Values within each column (c–e, d–f) or in the same row (a–c, b–d, a–e, b–f, g–h, i–j) with different superscripts are significantly different: c–e (P<0.005); a–e, a–c, b–d, b–f, d–f, g–h and i–j (P<0.0001) (χ^2-test).

Acknowledgments

The authors are grateful to Dr. T. Nagai for critical reading and editing of the manuscript.

References

1) Niwa, K. and Ohgoda, O. (1988): Synergistic effect of caffeine and heparin on *in-vitro* fertilization of cattle oocytes matured in culture. Theriogenology, 30, 733–741.
2) Ohgoda, O., Niwa, K., Yuhara, M., Takahashi, S. and Kanoya, K. (1987): Variations in penetration rates *in vitro* of bovine follicular oocytes do not reflect conception rates after artificial insemination using frozen semen from different bulls. Theriogenology, 29, 1375–1381.
3) Takahashi, Y. and First, N.L. (1993): *In vitro* fertilization of bovine oocytes in the presence of theophylline. Anim. Reprod. Sci., 34, 1–18.
4) Trounson, A. (1992): The production of ruminant embryos *in vitro*. In: Clinical Trends and Basic Research in Animal Reproduction (Dieleman, S.J., Colenbrander, B., Booman, P. and Vanderlende, T., eds.), pp. 125–137, Elsevier Science Publishers B.V., Amsterdam.
5) Niwa, K., Ohgoda, O. and Park, C.K. (1992): Effect of glucose in medium with caffeine and/or heparin on *in-vitro* penetration of bovine oocytes matured in culture. 12th International Congress on Animal Reproduction. Free Communications numbers, 671–673.
6) Parrish, J.J., Susuko-Parrish, J.L. and First, N.L. (1985): Effect of heparin and chondroitin sulfate on the acrosome reaction and fertility of bovine sperm *in vitro*. Theriogenology, 24, 537–549.
7) Parrish, J.J., Susuko-Parrish, J.L., Leibfried-Rutledge, M.L., Critser, E.S., Eyestone W.H. and First, N.L. (1986): Bovine *in vitro* fertilization with frozen-thawed semen. Theriogenology, 25, 591–600.
8) Parrish, J.J., Krogenaes, A. and Susuko-Parrish, J.L. (1995): Effect of bovine sperm separation by either swim-up or percoll method on success of *in vitro* fertilization and early embryonic development. Theriogenology, 44, 859–869.
9) Brackett, B.G. and Oliphant, G. (1975): Capacitation of rabbit spermatozoa *in vitro*. Biol. Reprod., 12, 260–274.
10) Konishi, M. and Aoyagi, Y.(1994): Studies on synthetic medium for development into blastocyst of *in vitro* fertilized bovine oocytes. J. Reprod. Dev., 40, j1–j4 (in Japanese).
11) Goto, K., Kajihara, Y., Kosaka, S., Koba, M., Nakanishi, Y. and Ogawa, K. (1988): Pregnancies after co-culture of cumulus cells with bovine embryos derived from *in vitro* fertilization of *in vitro* matured follicular oocytes. J. Reprod. Fert., 83, 753–758.
12) Fukuda, Y., Ichikawa, M., Naito, K. and Toyoda, Y. (1990): Birth of normal calves resulting from bovine oocytes matured, fertilized, and cultured with cumulus cells *in vitro* up to blastocyst stage. Biol. Reprod., 41, 114–119.
13) Lu, K.H., Gordon, I., Chen, H.B., Gallagher, M. and McGovern, H. (1988): Birth of twins after transfer of cattle embryos produced by *in vitro* techniques. Vet. Rec., 122, 539–540.
14) Hanada, A., Shioya, Y. and Suzuki, T. (1986): Pregnancy and birth of embryos nonsurgically transferred after *in vitro* fertilization and *in vitro* maturation. Proc. 78th Jpn. Soc. Zootech. Sci., I-36, 18 (abstr, in Japanese).
15) Aoyagi, Y., Fujii, K., Iwazumi, Y., Furudate, M., Fukui, Y. and Ono, H. (1988): Effects of two treatments on semen from different bulls on *in vitro* fertilization results of bovine oocytes. Theriogenology, 30, 973–985.
16) Shi, D.S., Lu, K.H. and Gordon, I. (1990): Effects of bulls on fertilization of bovine oocytes and their subsequent development *in vitro*. Theriogenology, 30, 324 (abstr).
17) Hillery, F.L., Parrish, J.J. and First, N.L. (1990): Bull specific effect on fertilization and embryo development *in vitro*. Theriogenology, 33, 249 (abstr).
18) Ling, Z.J. and Lu, K.H. (1990): Frequency of cleavage and development *in vitro* of bovine oocytes fertilized in different numbers in drops with different sperm concentrations. Theriogenology, 33, 275 (abstr).
19) Otoi, T., Tachikawa, S., Kondo, S. and Suzuki, T. (1993): Effects of different lots of semen from the same bull on *in vitro* development of bovine oocytes fertilized *in vitro*. Theriogenology, 39, 713–718.
20) Long, C.R., Damiani, P., Pinto-Correia, C., MacLean, R.A., Duby, R.T. and Robl, J. M. (1994): Morphology and subsequent development in culture of bovine oocytes matured *in vitro* under various conditions of fertilization. J. Reprod. Fert., 102, 361–369.
21) Fukui, Y., Fukushima, M. and Ono, H. (1983): Fertilization *in vitro* of bovine oocytes after various sperm procedures. Theriogenology, 20, 651–660.
22) Hanada, A. (1985): *In vitro* fertilization in cattle. — with particular reference to sperm capacitation by ionophore A23187. Jpn. J. Anim. Reprod., 31, 56–61 (in Japanese).
23) Parrish, J.J., Susuko-Parrish, J.L., Winer, M.A. and First, N.L. (1988): Capacitation of bovine sperm by heparin. Biol. Reprod., 38, 1171–1180.