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The efficacy of gradient Percoll® on bull sperm separation for *in vitro* fertilization

Marko Samardžija[1]*, Tomislav Dobranić[1], Martina Karadjole[1], Iva Getz[1], Silvijo Vince[1], Damjan Gračner[2], Nino Maćešić[1], and Ivana Filaković[3]

[1]Clinic for Obstetrics and Reproduction, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

[2]Clinic for Internal Diseases, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

[3]Belupo d.d. - Drugs and Cosmetics,Koprivnica, Croatia

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ABSTRACT

In the present study we examined the effect of density gradient preparation Percoll® on bull sperm separation and *in vitro* fertilization (IVF) results. Frozen/thawed semen from 5 Simmental bulls was pooled and sperm quality parameters were evaluated before and after sperm separation using Percoll® density gradient preparation. We evaluated sperm motility (subjective), sperm concentration (Thoma chamber) and sperm membrane activity by HOS assay. Initial sperm parameters results were as follows: motility 50%, sperm concentration 80.80 x 10^6 spz/mL and functional integrity of sperm cells 39.25%. Final sperm motility was 63%, final sperm concentration 28 x 10^6 spz/mL and HOS assay results after Percoll® gradient was 57.60%. A total of 211 oocytes were matured and fertilized *in vitro* and cultured in SOFaaBSA in five repetitions. The cleavage (D2), blastocysts (D7) and hatched blastocysts (D10) were 63.13%, 20.38%, and 10.64%, respectively. We found no significant correlations between sperm parameters and IVF results ($r<0.5$; $P>0.05$). It is possible to conclude that sperm evaluation parameters after sperm separation by gradient Percoll® are not reliable in predicting the outcome of IVF. However, using gradient Percoll® for sperm separation in IVF is necessary to improve the quality of separated sperm able to significantly improve sperm quality with a high rate of progressive motility and morphological normal spermatozoa.

Key words: bull, sperm, fertilization, Percoll®

Introduction

Sperm separation procedures are able to significantly improve sperm quality with a high rate of progressive motility and morphological normal spermatozoa. For this reason, in the

* Contact address:
Marko Samardžija, Clinic for Obstetrics and Reproduction, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000 Zagreb, Croatia, Phone: +385 1 2390 169; Fax: +385 1 2390 320; E-mail: smarko@vef.hr

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IVF, sperm separation methods has a very important role. Such selection of spermatozoa separates motile sperm from non-motile, removes seminal plasma, cryoprotective agents, other background materials and debris (ZAVOS, 1992) while also at the same time initiates capacitation of sperm (CENTOLA et al., 1998). Some of the most important sperm separation methods are: dilution and washing (FUKUDA et al., 1990), selective fractionation of subpopulations (density-gradient centrifugation): Percoll® (AVERY and GREVE, 1995), Bovipure® (SAMARDŽIJA et al., 2004); selective filters: Sephadex, glass wool (TIBARY et al., 1992); self-migration techniques (ROSENKRANZ and HOLZMANN, 1997). Percoll® is a medium for density-gradient centrifugation of cells, viruses and subcellular particles for research purposes. It is composed of colloidal silica particles (15-30 nm in diameter) coated with non-dialysable polyvinylpyrolidone (PVP). Percoll® density-gradient fractionation clearly separates spermatozoa from foreign material such as extender particles, cells and bacteria. The morphological selection of spermatozoa in the prepared population varies, with most tail, and mid-piece defects being primarily excluded (RODRIGUEZ-MARTINEZ et al., 1997).

Control of sperm quality after commercial freezing/thawing of bull semen is still restricted to the subjective assessment of sperm motility, despite its low correlation to fertility (SÖDERQUIST et al., 1991; KJAESTAD et al., 1993).

CORREA and ZAVOS (1994) used the Hypoosmotic swelling (HOS) test for evaluation of the functional integrity of frozen/thawed bovine sperm membrane. During the HOS test, functionally active membranes will undergo swelling and subsequently increase in volume to establish an equilibrium between the fluid compartment within the spermatozoa and the extracellular environment (JEYENDRAN et al., 1985). The osmolarity of the solution must be sufficient to produce the highest effect without resulting in the lysis of the cell membrane (ROTA et al., 1999).

The aim of this study was to discover the efficacy of gradient Percoll® on bull sperm separation for *in vitro* fertilization, and relevance of different sperm quality parameters to predict bovine *in vitro* fertilization.

Material and methods

General approach. For the purposes of our research a group of 5 Simmental bulls was chosen. Their non-return (NR) rate in 2002 was over 50% in more than 1000 fertile cows. The frozen/thawed sperm of all 5 bulls was mixed, making a pool. Sperm parameters were then estimated. All the components of the media for IVM/IVF/IVC used in this investigation were supplied by Sigma Chemical Co. (St. Louis, MO, USA).

Percoll® gradient. Percoll® gradient (Pharmacia, Uppsala, Sweden) was made according to PARRISH et al. (1995). Isotonic Percoll® solution was used for preparation of 90% and 45% gradients with HEPES-TALP medium. The Percoll® density gradient was

made by layering 1.5 mL of 45% Percoll® solution on 1.5 mL of 90% solution in 15 mL Falcon® tubes. On the top of the gradient 400 µL of thawed semen was layered and then tubes were centrifuged for 15 min at 700 g. The pellets were resuspended in the same amount of HEPES-TALP media and centrifuged for 7.5 min at 300 g. Afterwards, the pellets were resuspended in mIVF and the final concentration was adjusted to 1×10^6 spz/mL.

Sperm quality parameters asssessment. Sperm quality parameters were evaluated after thawing and after sperm preparation for IVF. Sperm concentration was determined with a Thoma chamber according to (HERAK, 1991). Progressive motility of semen was subjectively assessed by visual estimation under a microscope according to (HAMMMERSTEDT et al., 1988).

The functional integrity of bovine sperm membrane was determined by hypoosmotic swelling test (HOS). A hypoosmotic solution was prepared according to JEYENDRAN et al. (1984). The assay was performed by mixing 50 µL of semen with 1 mL of hypoosmotic solution and incubating at 37 °C for 60 min. A total of 400 cells were evaluated by including at least 5 different fields under a microscope at x 400 magnification.

Collection of cumulus-oocyte-complexes (COC) and in vitro maturation (IVM). Ovaries were collected from cows within 2 hours after slaughter and transported to the laboratory in sodium chloride solution (0.9%) with atb (100 i. u. penicillin and 100 µg streptomycin/mL) at 37 °C. Immature bovine oocytes were aspirated from 2 to 8 mm diameter follicles using 18G needles attached to a vacuum pump. Only oocytes with homogenous ooplasm and intact cumulus investment were selected for further procedures. They were washed twice in TCM 199 medium buffered with 15 mM HEPES supplemented with 10% of FCS, and then twice in IVM medium. Cumulus-oocyte complexes were matured *in vitro* in TCM 199 bicarbonate medium supplemented with 10% FCS, FSH/LH (Pergonal® 75/75 I.U., Serono), 1 µg/mL estradiol-17β and 100 µM cysteamine. Oocytes were incubated in 50 µL droplets of maturation media under mineral oil at 39 °C and with 5% CO_2 for 24 hours.

In vitro fertilization and culture (IVF and IVC). The expanded COCs were washed in TALP-HEPES medium supplemented with 3 µg/mL BSA-FAF and transferred in 40 µL droplets of IVF medium under mineral oil. The IVF medium was modified Tyrode˘s bicarbonate buffered solution supplemented with 10 µg/mL heparin, 0.5 µg/mL hypotaurine, 0.5 µg/mL epinephrine and 6 mg/mL BSA. The sperm suspension was then added at a volume of 10 µL to the droplets with oocytes. After sperm-oocytes co-incubation at 39 °C and with 5% CO_2 for 18 to 24 hours, the fertilized oocytes were denuded from cumulus cells and sperms and washed three times in HEPES-TALP medium and in culture medium. Synthetic Oviduct Fluid with amino acids and 8 mg/mL BSA, according to EDWARDS et al. (1997) was used. Fertilized oocytes were *in vitro* cultured in SOF medium without glucose for 48 hours and then transferred in SOF with 1.5 mM glucose and cultured *in vitro* until day 10 at 39 °C in 5% CO_2, 7% O_2 and 88% N_2, according to FURNUS et al. (1997). The

medium was changed every second day. Bovine embryos were evaluated according to IETS standards: on day 2 of culture we registered the number of cleaved embryos (fertilization rate); on day 7 the number of morulas and blastocysts, and on day 10 the number of hatched blastocysts (MANUAL of IETS, 1998).

Statistical Analyses. Statistical comparison was done by ANOVA (StatSoft, Statistica, 5.1 version 1984-1996.) using the arcsin transformation (arcsin$\sqrt{}$ P/100) of the percentage values. We also used Sheefe's tests post-hoc analysis and we found the correlations between sperm parameters and IVF results.

Results

The mean result of the initial sperm motility was 50 ± 4.47%. Concentration values were between 78×10^6 spz/mL to 83×10^6 spz/mL or an average of 80.80 ± 0.97 x 10^6 spz/mL. The HOS test results values were between 28.77% and 50.22% of the sperms with functional intact plasma membrane. The mean value of the HOS test was 39.25 ± 3.63% (Table 1.).

Table 1. Initial sperm parameters results after thawing

Nº of replication	Initial motility %	Initial concentration 10^6/mL	HOS % positive
1	40.00	80.00	34.77
2	40.00	80.00	28.77
3	60.00	83.00	39.73
4	60.00	83.00	50.22
5	50.00	78.00	42.76
Mean	50.00	80.80	39.25
Var.	100.00	4.70	65.70
SD	10.00	2.17	8.11
SEM	4.47	0.97	3.63

After sperm separation on gradient Percoll® motility values were between 50 to 75%, or an average of 63 ± 4.36%. Sperm concentration was 28 ± 1,82 x 10^6/mL. HOS test results values were between 51.84% and 63.72% of the sperms with functional intact plasma membrane. Mean value of the HOS test was 57.60 ± 2.04% (Table 2.).

A total of 211 oocytes were matured and fertilized *in vitro* and cultured in SOFaaBSA in 6 repetitions. Oocytes cleavage results were 63.13 ± 2.92%. The percentage of morulas and blastocysts on day 7 of the culture was 20.38 ± 2.95%. Results of hatched blastocysts on day 10 of culture were 10.64 ± 2.83%. (Table 3).

Table 2. Sperm parameters results after gradient Percoll®

Nº of replication	Final motilty %	Final concentration 10^6/mL	HOS % positive
1	50.00	25.00	51.84
2	60.00	26.00	56.92
3	60.00	28.00	55.31
4	70.00	26.00	63.72
5	75.00	35.00	60.19
Mean	63.00	28.00	57.60
Var.	90.00	16.50	20.76
SD	9.75	4.06	4.56
SEM	4.36	1.82	2.04

Table 3. *In vitro* fertilization and *in vitro* culture results

Nº of replication	total	% cleveage day 2	% M+B1 day 7	% HBL day 10
1	35.00	68.57	22.86	8.57
2	47.00	61.70	12.77	8.51
3	50.00	60.00	14.00	6.00
4	55.00	54.55	27.27	21.82
5	24.00	70.83	25.00	8.33
Mean	42.20	63.13	20.38	10.64
Var.	157.70	43.59	43.40.	40.17
SD	12.56	6.60	6.59	6.34
SEM	5.62	2.92	2.95	2.83

Discussion

Until recently, techniques of the centrifugation method on gradient of Percoll® were the most widely used methods in the preparation of bull sperm for the purpose of *in vitro* fertilization (MENDES et al., 2003). The problem is that some batches of Percoll® have an endotoxic effect. It was therefore discarded for use in assisted reproduction techniques in human medicine (CHEN and BONGSO, 1999). There have been reports that batches of Percoll® differ in composition and this variation may affect cleavage rates and

embryo development (MENDES et al., 2003). In our investigation we assumed that there were significant differences ($P<0.01$) between the final and initial sperm results. This is congruent with results obtained by CORREA and ZAVOS (1996) and MATKOVIĆ et al. (2002) for the same method. CORREA and ZAVOS (1996) used appliance gradient Percoll® and they found that sperm motility between 74-78%, which is different from our results for sperm motility after gradient Percoll® of 63%. We assume that this was expected because they had higher initial values of motility from ours after thawing sperm. Results congruent to ours were obtained by TANGHE et al. (2002) who investigated the Percoll® method and obtained motility of 68% for a first group consisting of 3 bulls, but weaker motility (52%) for a second group consisting of 3 other bulls. Our results for the HOS test performed after Percoll® yielded 57.60% of sperm cells with functional active plasma membrane. CORREA and ZAVOS (1996) yielded 41-46% and MATKOVIĆ et al. (2002) obtained 53% of sperm cells with functionally active plasma membrane using the Percoll® method. A high, positive and significant correlation was found between the IVF results ($r>0.5$; $P<0.05$), but there was no correlation ($r<0.5$; $P>0.05$) between the sperm evaluation parameters and IVF results. This result was congruent with the results of DOWSETT and PATTIE (1982). The mentioned authors found no significant correlation between sperm motility and the IVF results. Our results of IVF and IVC were at average levels for gradient Percoll®, and we did not confirm thesis of KJAESTAD et al. (1993) and ZHANG et al. (1998) who found that different sperm parameters such as percentage of live sperms, percentage of progressive motility sperms and acrosome status, represent significant correlations with sperm ability to fertilize oocyte.

It is possible to conclude that sperm evaluation parameters after sperm separation by gradient Percoll® are not reliable in predicting the outcome of IVF. However, using gradient Percoll® for sperm separation in IVF is necessary to improve quality of separated sperm.

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SAŽETAK

Istraživanjem pripreme sperme za *in vitro* oplodnju na gradijentu Percoll®, odabrana je skupina od 5 bikova simentalske pasmine. Duboko smrznuta i odmrznuta sperma svih 5 bikova bila je pomiješana i ocijenjena netom nakon odmrzavanja i nakon pripreme Percoll®-om. Ocijenjena je pokretljivost (subjektivno), koncentracija (Thomina komorica) i funkcionalna cjelovitost membrane spermija HOS testom. Početna pokretljivost iznosila je 50%, koncentracija 80,80×10^6 spz/mL i poluèeno je 39,25% spermija s funkcionalno neoštećenim plazminim membranama. Završna pokretljivost spermija iznosila je 63%, a koncentracija 28×10^6 spz/mL spermija. Rezultati HOS testa nakon gradijenta Percoll® iznosili su 57,60% spermija s funkcionalno neoštećenim plazminim membranama. Za oplodnju jajnih stanica spermom pripremljenom Percoll®-om nakon postupka aspiracije folikula te dozrijevanja jajnih stanica *in vitro* u SOFaaBSA mediju dobiveno je ukupno 211 jajnih stanica u 5 ponavljanja. Rezultati brazdanja jajnih stanica iznosili su 63,13%, morula i blastocista sedmoga dana 20,38%, a izvaljenih blastocista 10-og dana uzgoja 10,64%. Nismo ustanovili visoku, značajnu korelaciju između značajki sperme s rezultatima oplodnje *in vitro* (r<0,5;P >0,05). Možemo zaključiti da ocjenjivani parametri kvalitete sperme nakon pripreme za IVF gradijentom Percoll® nisu pouzdani pokazatelji uspjeha IVF-a. Međutim, korištenje gradijenta Percoll® za pripremu sperme u postupcima IVF-a neophodno je u smislu poboljšanja kvalitete izdvojene sperme.

Ključne riječi: bik, sperma, Percoll