COMPARATIVE BOAR AND BULL SEMEN EVALUATION AFTER PERCOLL TREATMENT

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Abstract
There are numerous methods used in IVF both in bovine and pig for sperm separation and Percoll gradient is one of them. After using Percoll gradient spermatic parameters were as following: motility increased to 78.33% in boar samples and to 75% in bull samples; the number of live spermatozoa increased with 42.91%, 31.46% and 21.88% in boar sample, respectively with 19.37%, 9.34% and 5.85% in bull samples; normal spermatozoa increase to 98.52-98.92% in boar and to 84.22-93.97% in bull samples. Acrosome integrity, another parameter studied, indicate that in bull sample intact acrosome was before 83.97%, 87.58% and 88.1%, respectively 91.39%, 93.11% and 92.56% after using Percoll gradient, similar increase was in boar sample to 95.49%-98.42%. Therefore, Percoll is an easier and fast way to select viable and normal spermatozoa for IVF techniques
Keywords: *Percoll gradient, spermatic parameters, IVF*

In assisted reproduction techniques (ART) , used for research and practical purposes in animal field, both gametes quality have a great importance. Sperm separation methods have an important role in ART. These methods suppose selection of motile spermatozoa from nonmotile, removing seminal plasma, cryoprotective agents other debris and materials, and also to initiate the capacitation of the sperm.

In bovine ART there are used some methods for sperm separation such as Percoll, BoviPure and swim-up. Percoll and BoviPure are based on density gradient centrifugation and swim-up is based on motility spermatozoa selection.

Percoll is one commercial medium for the density-gradient centrifugation of cells, organelles, viruses and other subcellular particles. Percoll is especially useful as a first step to enrich for cell populations before attempting finer resolution or extraction of nucleic acids. Since its introduction in 1977 it has become the choice of researchers worldwide. Percoll is composed of colloidal silica particles (15-30 nm in diameter) coated with nondialysable polyvinylpirolidone (PVP). BoviPure is an iso-osmotic salt solution containing colloidal silica particles coated with silane. (11).

There are numerous studies both in bovine and swine reproduction regarding the effects of gradient Percoll on sperm preparation for IVF. In pigs, Noguchi et al. (2013) obtained significantly higher percentages of motile sperm, sperm with intact plasma and acrosome membranes, rates of penetration, cleavage and blastocyst formation after Percoll separation than simple centrifugation (8). In bovine, Samardzija et al. (2006) didn't find significant differences regarding sperm evaluation parameters between Percoll and BoviPure, although cleavage and blastocysts rate were significantly higher for the BoviPure group, the number of hatched blastocysts did not differ (11).

The purpose of this research was to evaluate the effect of Percoll gradient on some spermatic parameters (motility, viability, morphology, acrosome integrity) both in bull and boar.

Materials and methods

We used various solutions, either purchased or prepared in our laboratory. As a base for cell culture we have chosen Earl`s solution (1943) prepared in our laboratory following the

original receipt (for 100 ml solution we used Sigma products: 0.0265 g CaCl2x2H2O, 0.02 g MgSO4x7H2O, 0.04 g KCl, 0.68 g NaCl, 0.01586 NaH2PO4x2H2O, 0.1 g C6H12O6, 0.0011 Phenol Red and 0.22 g NaHCO3). All ingredients were solubilized in ultrapure water (Millipore) and subsequently sterilized by filtering with 22 μm filters (Millex GS Filter Unit) and kept at 4ºC until using. Initially a Earl 10X solution was prepared and for obtaining Earl 1X, 10 ml of Earl 10X were mixed with 90 ml ultrapure water and 0.22 g of NaHCO3.

Percoll stock solution was obtained by mixing 9 ml of Percoll with 1 ml Earl 10X. In order to avoid precipitation, the solutions were mixed through a continuous and very gentle stirring. There were necessary multiple attempts in order to obtain a perfect crystal clear solution. For gradient concentration centrifugation, were also prepared Percoll 90% and 45% by dilution with Earl 1X.

Bull semen sample consisted in three straws from bulls: Maradona, Heynckers and Bonaqua. The straws were thawed at 37ºC for one minute and their content was subsequently analyzed.

Boar semen sample consisted in three probes: Pietrain breed, ID boar 82000, Marele Alb, ID boar 88177 and Duroc breed, ID boar 689/33.

Mobility of spermatozoa was examined with a decimal system (Bara, 2012). Its viability was estimated following the eosine-negrosine (Vital Screen) staining. Morphological features of the semen were estimated subsequently to Spermac staining, using a 40X objective (Leica M3350).

There is no unanimously accepted Percoll protocol for use in veterinary assisted reproduction. The method principle consists of semen centrifugation in a concentration gradient followed by precipitate sampling and semen concentration, morphology, viability and motility assessment. 200 μl of bull semen, respectively 2000 μl of boar semen were slowly introduced in the centrifuge tube already containing Percoll 90% - 45% previously warmed at 37ºC for four hours. The mixture was centrifuged (Hettich 350R) at 600g for 20 minutes. The supernatant was removed and 2 ml of EarlX1 solution was added, than the mixture was centrifuged another 20 minutes at 200g. Again, the supernatant was removed and 700 μl of Earl 1X were added, followed by semen assessment.

Results and discussions

After using Percoll gradient occurs an increase in the number of motile spermatozoa for all samples examined (figure 1). When analyzing boar semen, the highest values were obtained from Large White boar (boar ID 88177), mobility has increased by 20%, and in Duroc breed (ID boar 689/33) with 35%. For the first sample, Pietrain breed (boar ID 82000) the mobility increased by 5% after using Percoll gradient. In average motility increased with 20% in boar samples. Also in bull sperm analyze, motility increased after using Percoll gradient with 5-10%.

In average motility increased to 78.33% in boar samples and to 75% in bull samples. A good boar semen should have during examination at least 70% motility. Due to fact that pig oocytes maturation time is around 48h, boar samples were examined after Percoll using also at 48h, and the motility was still high at 73.33% in average.

The data obtained show the importance of using Percoll gradient in the selection of mobile sperm for later use in assisted reproductive techniques such as in vitro fertilization and intracytoplasmic sperm injection (ICSI).

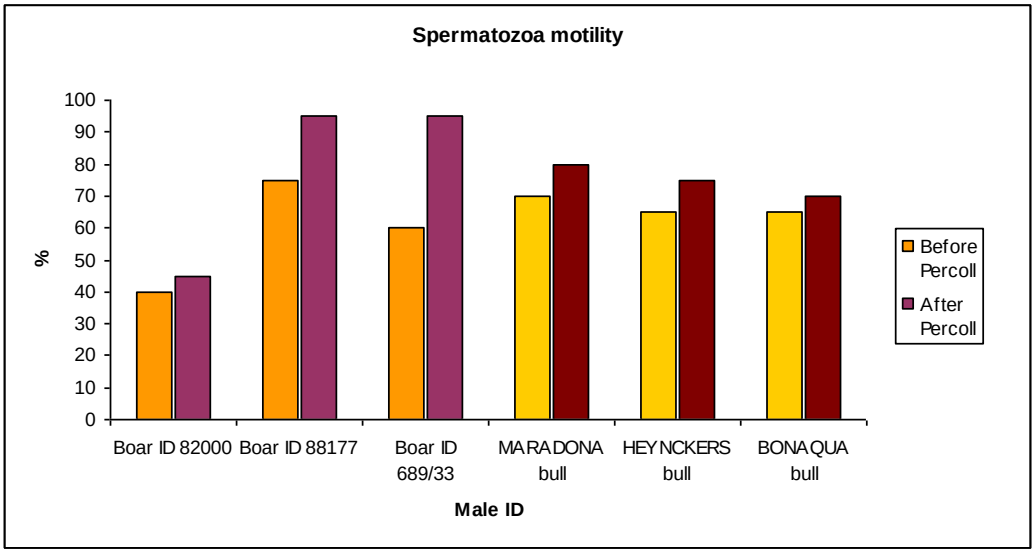

Figure 1. Boar and bull spermatozoa's motility before and after using Percoll gradient

Similar results on boar semen analysis were obtained by Grant et al. (1994), so the use of centrifugation the gradient discontinuous Percoll in the preparation of sperm boar for *in vitro* fertilization resulted in obtaining sperm with mobility and movements characteristic significantly higher ($p < 0.0001$) compared to the group prepared by simply washing. In vitro matured oocytes fertilized with sperm selected by gradient Percoll had cleavage rates significantly higher ($p < 0.0001$), although electronomicroscopic investigations did not reveal ultra-structural differences between groups. Similar results were reported also by Ding et al. (2000)(2).

Studies in pig IVF have shown that almost every studied parameter of boar semen in significantly different between penetration successes and failures, but most of them are interrelated, which emphasize the complexity of sperm functions and the difficulties in assessing boar fertilizing ability .

IVF systems can be used successfully for evaluating the fertilizing capacity and are more accurate than other methods.(3)

From figure 2 it can be seen a positive correlation between motility and viability, thus the number of live sperm after using Percoll gradient has increased for all samples, with 42.91%, 31.46% and 21.88% in boar sample, respectively with 19.37%, 9.34% and 5.85% in bull samples. Similar results analyzing bull semen were obtained by Mircu et al. (2015), Percoll raised the percentage of viable sperm to 40-56%.

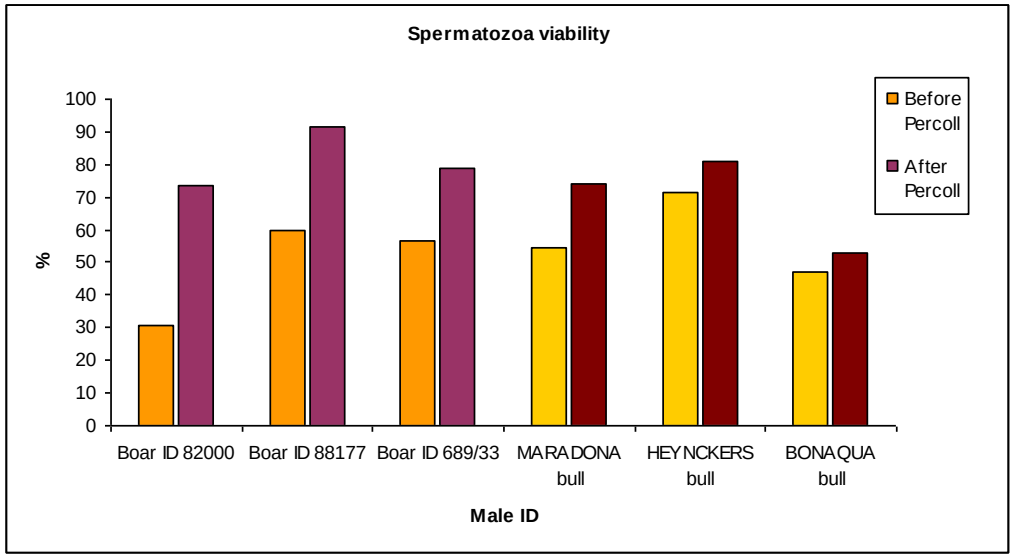

Figure 2. Boar and bull spermatozoa's viability before and after using Percoll gradient

Comparing Percoll gradient with Swim-up, another method used for viable sperm selection in bovine ART researchers obtained a large number of viable spermatozoa with intact acrosome subsequent to Percoll use. The difference of percentage for viable spermatozoa could be explained through centrifuge force action which can affect motility and sperm membrane integrity (Verberckmoes et al., 2000) and also by Swim-up method principle which relies on spermatozoa movements (7, 12). Percoll gradient selects sperm based on their density and is not a physiological mean of separating viable spermatozoa. In cattle more sperm were recovered with Percoll gradient than swim-up, however penetration rate was higher with swim-up separated sperm (Parrish et al., 1995). Similar results were obtain in buffalo (*Bubalus bubalis*) sperm research by Mehmood et al. (2008). They obtained significantly higher motility and greater IVF rate (cleavage rate and cleavage index) with swim-up method, although Percoll gradient separated greater number (6).

Regarding morphology in boar samples, the normal spermatozoa ranged between 34.29-94.05%, while anomalies vary between 5.45-65.71%., and increase to 98.52-98.92%, except sample ID 82000 were the number was lower after Percoll gradient. It can be seen, in figure 3 that the use of Percoll gradient does not increase significantly the number of normal spermatozoa. In bull samples normal spermatozoa increase to 84.22-93.97%.

Similar results in boar were obtained Matas et al. (2011). Thus the use of discontinuous Percoll gradient centrifugation 45/90 increased the sperm with normal morphology to values over 95% compared to 82% as was recorded in the control group. This decrease of sperm with anomaly is given by the spermatozoa with cytoplasmic drop (immature) (low density) and those with defective tail. Having a lower density, they remain in 45 gradient, thus being eliminated from the sample (5).

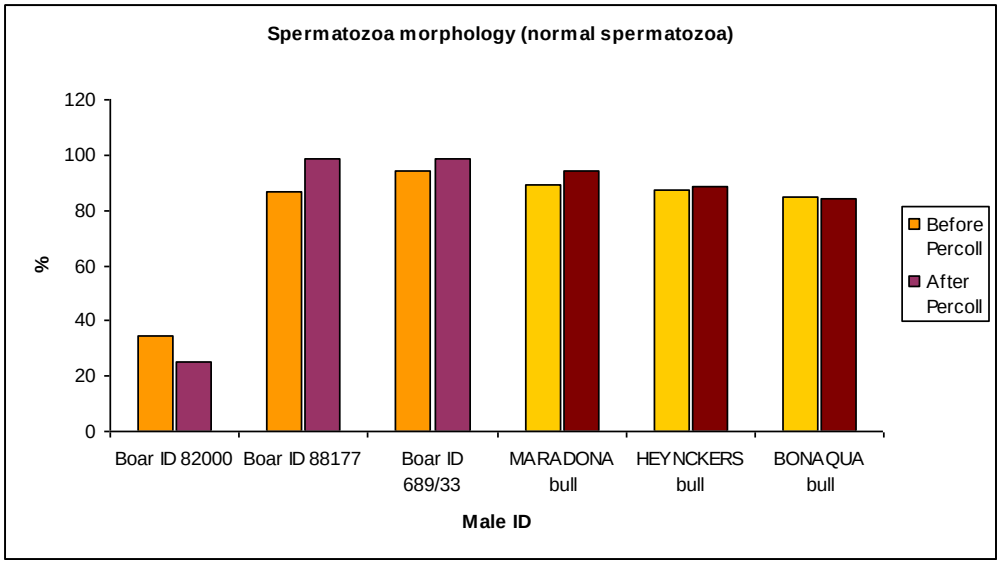

Figure 3. Boar and bull spermatozoa's morphology before and after using Percoll gradient (spermatozoa with normal aspect)

An intact acrosome is required for oocyte fertilization, so his integrity is vital to achieve optimum fertilization. If the percentage of acrosome-intact sperm is low, then fertility may be compromised. Acrosome reaction is seen as multiple fusions between outer acrosomal membrane and plasma membrane at the anterior region of sperm head, extensive formation of hybrid membrane vesicles and exposure of inner acrosomal membrane and acrosomal contents (11,14).

Studies indicate that acrosome integrity assessment provides a better characterization of sperm even to mobility. It is considered a good ejaculate if acrosome integrity is more than 51%. The same principles applied to mobility and morphology is valid for acrosome integrity, so if semen is stored for a period of time, this parameter decreases (10).

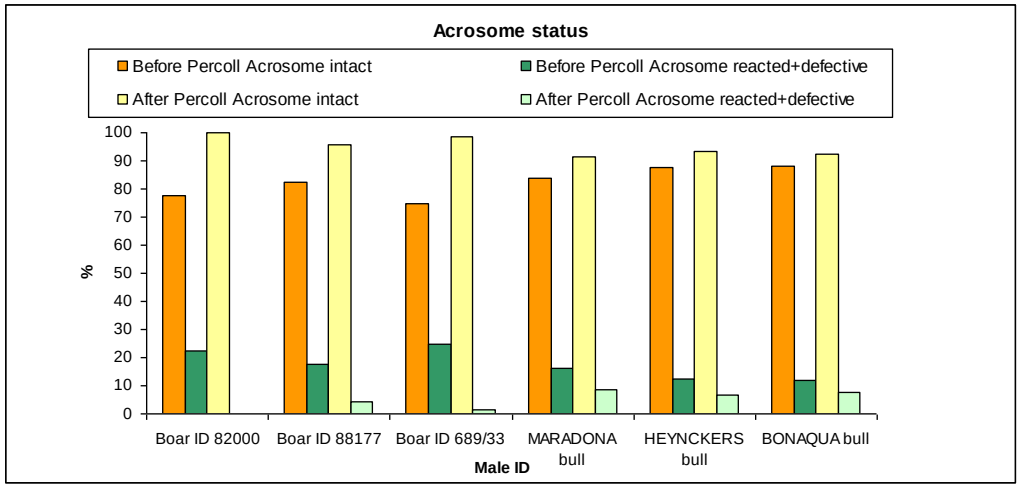

Figure 4. Boar and bull spermatozoa's acrosome analysis before and after using Percoll gradient

The results obtained by Noguchi et al., (2013) points out that the head of the sperm membrane integrity after using Percoll is more important in the development of the embryo *in vitro* than mobility. Also the percentage of motile spermatozoa and acrosome intact after using Percoll gradient was significantly higher than those obtained simply by centrifugation; and fertilization rate after IVF blastocyst formation were significantly enhanced separation on Percoll gradient and were positively influenced by intact membranes of the sperm head (8).

It can be seen in Figure 4 a reduction in the number of spermatozoa with reacted and defective acrosome, which indicates that by using Percoll gradient 45/90 we obtain a higher percentage of spermatozoa with intact acrosome, spermatozoa that can achieve fertilization. The same modification are observed in bull sample, so before Percoll spermatozoa with intact acrosome was 83.97%, 87.58% and 88.1%, respectively 91.39%, 93.11% and 92.56% after using Percoll gradient.

Conclusions

Spermatic parameters (motility, viability, morphology, acrosome integrity) analyzed in bull and boar semen samples after using Percoll gradient were higher than before.

Percoll is an easier and faster way to select viable and normal spermatozoa for IVF techniques.

Percoll gradient along IVF systems in bovine and pig reproduction can be used successfully for accurate evaluation of the sperm fertilizing capacity.

Aknowlegdements

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