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Separation of Motile Bovine Spermatozoa for In Vitro Fertilization by Electrical Charge

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Abstract

Objective: Sperm selection is essential to Assisted Reproductive Technology (ART), influencing treatment outcomes and the health of the resulting offspring. Several techniques have been developed to recover a homogeneous population of highly motile sperm cells, including centrifugation gradients, swim up and filtration procedures. It is known that the head of spermatozoa has a negative electric charge, and several studies have tried to use this physiological characteristic for the selection of high quality motile sperm from frozen-thawed semen. The study aim was to design a device that would be able to separate viable, high quality sperm for IVF after thawing, using electrical characteristics of sperm.

Methods: Samples of the semen solution were taken at different times from both the anode and cathode areas. The electric charges used were 0, 1, 5 and 10 volts (V), at times of 0, 5, 10 and 20 minutes. In the second phase of the experiment, to evaluate the ability of the selected sperm to fertilize oocytes, we fertilized oocytes with semen processed with either the EC (10 volts per 10 minutes) and the discontinuous gradients.

Results: Concentration and motility were affected by the voltage: V0 was different from V1, V5 and V10 ($P<0.0001$). There were no differences when we compared the electric channel (EC) to the Percoll discontinuous gradient on cleavage rate and embryo development rate. To verify the effect of magnetic field on the ratio of sex we evaluated the embryo sex distribution: we did not find any effect on embryo sex ratio.

Conclusion: In conclusion all these observations may allow for the streamlining of many IVF protocols. Further, the decreased time gametes are outside of the optimal environment of the incubator may potentially reduce the stress on the gametes during the IVF procedures. Future, work will focus on pregnancy rates of embryos produced with EC separated sperm after IVF.

Keywords: Bovine IVF; Oocyte maturation; NMR; Electrical charge; Sperm; Semen selection; ART outcomes

Introduction

Frozen-thawed semen has been extensively used for bovine artificial insemination (AI) and in vitro fertilization (IVF) procedures [1]. Most spermatozoa are injured during semen freezing and thawing processes leading to reduction in sperm motility and to membrane structure [2]. The presence of non-motile or damaged sperm in the frozen semen negatively affects semen quality; motile, morphologically normal sperm must be separated from seminal plasma components, the extender supplements and/or undesired cells, by means of a selection technique. In cattle, higher pregnancy rates were obtained with selected sperm compared to unselected samples from the same ejaculate, when equal numbers of motile cells were used for insemination [3]. Thus, sperm treatments to select motile spermatozoa from frozen–thawed semen are essential when in vitro fertilization (IVF) is performed in cattle [4]. Several techniques have been developed to recover a homogeneous population of highly motile sperm cells. These techniques are used for removing seminal plasma, dead cells, abnormal sperm, cryoprotective agents, and other factors [5]. The techniques include Percoll density gradient centrifugation, swim-up migration, washing by centrifugation, glass wool filtration and several others [6-12]. Acrosome integrity, as well as enzyme maintenance, is crucial for successful fertilization [13,14]. When the acrosomal membrane is disrupted, the penetration of specific molecules is facilitated, allowing for the detection of damaged acrosomes [15,16]. Percoll centrifugation is recognized as one of the easy and common methods for separating spermatozoa [11,17] and is the most widely used in bovine IVF laboratories [4,7,9]. Oliveira et al. [5,18] reported that the percentage of sperm cells with intact acrosomal membranes was reduced from 85.0% (bovine frozen-thawed semen) to 45.1% following Percoll centrifugation. In contrast, Machado et al. [9], and Somfai et al. [19] reported that the proportion of frozen-thawed bull sperm with intact acrosomes increased after centrifugation using a gradient of 2 mL of 45% over 2 mL of 90% Percoll solution. Perhaps the differences in Percoll layers between these studies could be an explanation for the divergence in results regarding the acrosomal status after centrifugation. This finding reinforces the idea that PVP (polyvinylpyrrolidone) present in silica-based density gradients can be damaging to sperm membranes [17]. In human

sperm, PVP has been shown to cause plasma membrane, acrosome, and mid-piece damage without centrifugation [20]. In addition, the high sperm concentration within the pellet containing silica-based medium may have contributed to the occurrence of ultrastructural damage to sperm cells [21]. Others semen separation techniques available for removing undesirable spermatozoa, seminal plasma, cryoprotective agents, leukocytes and debris include Sephadex columns and glass wool filtration. These spermatozoa separation procedures have been characterized with human spermatozoa [21-25] and have also been evaluated for use with bovine spermatozoa [1,26,27].

In recent years, some researchers tried to take advantage of the electric potential of sperm in order to select the best sperm for IVF. Mature sperm possess an electric charge of −16 to −20 mV [28]. In a normal mature sperm, the membrane glycocalyx is rich in sialic acid [29]. High levels of sialic acid residues on the sperm membrane increase its net negative charge, which may play a role in capacitation and the formation of binding bridges between sperm membrane proteins and the oocyte [30]. The presence of high concentrations of sialic acid residue in the sperm's membrane may be indicative of normal spermatogenesis and maturation status of sperm. In addition Simon et al. showed that sperm with higher negative charge had a low rate of DNA damage [31]. So the aim of this study was to evaluate whether an electrical channel could be used to select the sperm for IVF. Currently, there are no reports in the literature regarding the selection of viable, high quality motile sperm using electric charge. The other studies using electric charge techniques were also able to select motionless sperm [31]. To examine this question, we have created a device containing an electrified channel, in which it was possible to change time and electric current. In the first experiment, we estimated the ability of sperm to move in direction of the positive electrode: by changing the voltage and the time of exposure. The second step was to evaluate the ability of the selected sperm to fertilize oocytes and then to determine the quantity and quality as well as the gender of the embryos produced. The evaluation of sex was considered as an important parameter of embryo quality. Further, this parameter was analyzed to evaluate the behavior of the sperm with different sex chromosomes (X or Y) in the electric field.

Materials and Methods

2.1. Reagents and Media

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (USA). The medium for electric channel (MEC) contained: NaCl 98.8 mM; KCl 3 mM; NaH_2PO_4 0.35 mM; $CaCl_22H_2O$; $MgCl_26H_2O$; $NaHCO_3$ 25 mM; HEPES 10 mM, plus 10 mg/mL of BSA. The pH and the osmolarity were respectively 7.4 and 280. The IVF medium was Tyrode's modified medium [32] without glucose and bovine serum albumin (BSA), supplemented with 5.3 SI/mL heparin, 30 µM penicillamine, 15 µM hypotaurine, 1 µM epinephrine, and 1% bovine serum (BS)(B9433). The IVC medium consisted of Synthetic Oviduct Fluid (SOF) medium [33], with 30 µL/mL essential amino acids, 10 µL/mL non-essential amino acids, and 5 % BS.

2.2. Device fabrication

The device, the electric channel (EC), was constructed in polydimethylsiloxane (PDMS, Syligard 185, Dow Corning Corp., Midland, MI) [34,35]. The polymer was mixed with its corresponding hardener, in a 10:1 ratio by weight, to create the PDMS mixture. Air bubbles were induced in the PDMS during mixing. The air was removed (degased) by placing the PMDS in an evacuated environment (desiccation chamber) for 45 minutes. After degassing, PDMS was placed on a hot plate at 60°C for 4 h. The electricity was produced using a power supply box for electrophoresis (BIORAD power Pac 3000) and a circuit consisting of titanium electrodes which were located in the channel (6 cm long x 0.5 cm deep x 0.5 cm wide) of PDMS polymer where the semen was placed as shown in Figure 1.

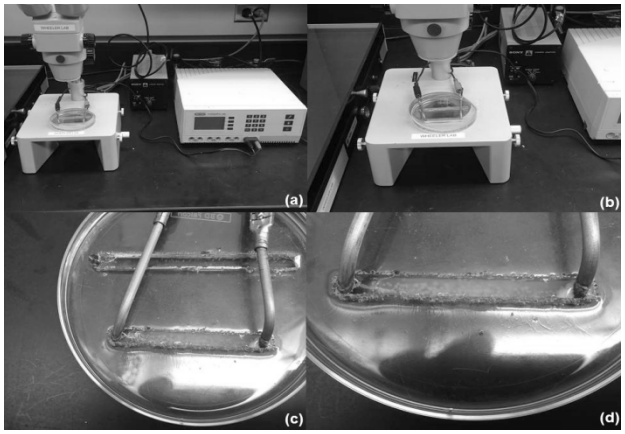

Figure 1: Photographs showing the Polydimethylsiloxane (PDMS) device and the equipment used to perform the sperm separation. a) Complete sperm separation set-up (microscope, PDMS device with titanium bar and electrical power source; b) Microscope, PDMS device with titanium bar; c) PDMS device detail with channel filled with sperm separation medium; d) PDMS device containing semen with electric charge being applied.

2.3. In vitro embryo production

The matured oocytes were purchased from DeSoto Biosciences (Seymour, TN, USA). In vitro matured cumulus-oocyte-complexes (COCs) were washed and transferred, 20-30 per well, into 300 µL of IVF medium covered with mineral oil. For each replicate, two straws of frozen semen (from bulls previously tested for IVF) were thawed at 37°C for 40 sec. The sample was divided in two samples: one part was processed via Percoll discontinuous gradient (45–80%) [36] and the other was processed via the electric channel (EC). The electricity method consists of one centrifugation at 800 RPM for 10 min to obtain the pellet, then the sample was deposition into the center of device and subjected to the electric current. After the electrical cycle, the sample was centrifuged for 5 minutes at 800 RPM. After processing pellets were diluted with IVF medium and added to the fertilization wells at the concentration of 1 x 10^6 sperm/mL. Gametes were co-incubated for 20 h at 39°C , in 5% CO_2 in air, after which presumptive zygotes were vortexed for 2 min to remove cumulus cells in HEPES-TCM with 5% BSA, washed twice in the same medium, and transferred, 30–50 per well, into 400 µL of SOF. Zygotes were incubated in a humidified mixture of 5% CO_2, 6% O_2 and 88% N_2 in air at the temperature of 39°C. The percentage of cleaved embryos and embryos reaching blastocysts were determined at day 7 of culture (day 0 = IVF day).

The embryos were scored for quality on the basis of morphological criteria, and only Grade 1 and 2 blastocysts (Bl) were considered in the evaluation of the final embryo yield.

2.4. Embryo sexing

Embryos were put into 10 µL of PBS, frozen and stored individually at -80°C until assay. Twenty µL of a lysis buffer containing 15 mM Tris-HCl pH 8.9, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, and 150 µg/mL proteinase K was added to each tube. The tubes were incubated at 55°C for 1 h, then proteinase K was inactivated by incubation at 90°C for 10 min. For each round of PCR, purified male DNA prepared from blood of adult cattle were used as positive controls (Wizard genomic DNA Purification Kit—Promega cat. A1125). Amplifications were performed by adding 12.5 µL of a PCR mix (JumpStart™ Taq ReadyMix™), two pairs of primers (1 µL for each pair), 1 µL of template DNA, and 10.5 µL of H_2O to each PCR tube for a total volume of 25 µL per tube. Each pair of primers was amplified individually. The first pair is specific to a sex-determining region of the bovine Y chromosome (SRY) [37], while the second pair of primers is specific to an autosomal gene (tRep-137) [36,38]. All samples were denatured at 95°C for 15 min, followed by 39 cycles consisting of denaturation at 96°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 45 s. After the last cycle, all samples were incubated for a further 5 min to assure complete extension. PCR products were analysed using a 2% agarose gel with 0.1 lg/ml of ethidium bromide in the gel. The resulting bands migrated by electrophoresis and the products were observed with UV transillumination. Each template was allocated in a single well so that each embryo corresponds to two adjacent wells. Therefore, the embryo was assessed as male when both bands were visible in the corresponding spots, and as female when in two adjacent wells only one band was visible, as shown in Figure 2.

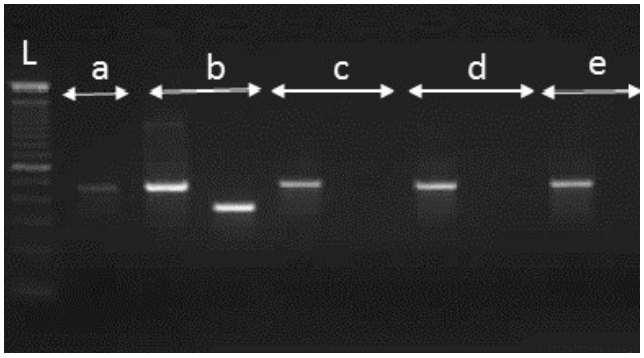

Figure 2: Photograph of a gel showing the separated PCR products after amplification of male and female specific DNA sequences from individual IVF embryos. Column legend: Lane L= Ladder; Lane a = Control; Lane b = Male embryo; Lanes c, d, e = Female embryos.

2.5. Experimental design

After thawing, semen was centrifuged at 800 rpm and 10 µL of pellet (10 µL with a sperm concentration of 20-25 x 10^6/ml) were positioned in the EC at the center of the electric current (1 mL of MEC). A ten µl sample of semen solution was taken at times 0, 5, 10, and 20 minutes from both the anode and cathode sites. These samples were then assessed for sperm concentration and sperm motility. The electric energy charges used were 0, 1, 5 and 10 volts (V). Three different bulls were used for this experiment, and for each bull 4 replicates were performed for a total of 12 replicates. The concentration was evaluated with a Burker chamber and motility was evaluated by observing the number of motile sperm in each chamber. In the second phase of the experiment, oocytes (n= 656) were fertilized with semen processed with either the EC or the Percoll gradient (Control). The percentage of cleaved embryos and embryos reaching blastocysts was determined at day 7 of the culture (day 0 = IVF day). The embryos were scored for quality on the basis of morphological criteria, and only Grade 1 and 2 blastocysts (Bl) were considered in the evaluation of the final embryo yield. Finally, representative samples of the blastocysts produced in different systems had their gender (sex) determined as previously described. This sexing experiment was repeated 5 times.

2.6 Statistical analyses – experiment 1

All recorded parameters were subjected to a two way analysis of variance using the procedure of the Generalized Linear Model (SAS, 9th version, 1999). Independent variables were the time of observation (t0,t1,t2,t3), the voltage (V0,V1,V5,V10), and the interaction between those variables. Data were normally distributed. Least square means tests were used to perform statistical multiple comparisons. The alpha level was set at 0.05. All data were expressed as quadratic means and mean standard errors.

An addition analysis was performed considering t2 as the optimal time (10 min after) to determine which voltage was more useful for sperm separation. In this case the recorded parameters were subjected to one way analysis of variance using the procedure of the Generalized Linear Model (SAS, 9th version, 1999), where the independent variable was the voltage (V0,V1,V5,V10) and concentration of sperm and sperm motility were the dependent variables.

2.7 Statistical analyses experiment 2

All recorded parameters were subjected to a Student's t-Test. The parameters compared were cleavage rate, blastocyst rate and the percentage of embryos on cleaved. The alpha level was set at 0.05. All data were expressed as quadratic means and mean standard errors.

Results

Experiment 1

The concentration of sperm at the negative anode was near to the zero so we evaluated only the cathode.

The effect of the time (df=3; F(3,3)= 19.22; P<0.0001), voltage (df=3; F(3,3)= 15.65; P<0.0001) and their interaction (df=9; F(3,3)= 3.44; P=0.0008) were significant on sperm concentration.

The concentration of sperm at the positive anode was affected by the time of observation, t0 was statistically different from t2 and t3 (P<0.0001), respectively, whereas t1 were statistically different from t2 (P=0.0013) and t3 (P<0.0001), respectively. There was no difference in the concentration between t2 and t3 (Table 1). Timing also had an effect on motility, where t0 was different from the others (P<0.0001) and t2 was different from t3 (p=0.02) (Table 1). Concentration was also affected by the voltage: V0 was different from V1 (P=0.02) and from V5 and V10 (P<0.0001). Concentration at V1 was 0.07±0.02 and it was different from V5 (0.18 ±0.02; P=0.0014) and from V10 (0.19 ±0.02; P=0.0004) (Table 2). Voltage affected motility where V0 was different from the others (P<0.0001) and v1 was different from v5 (p=0.02) (Table 2). To choose the best voltage we chose to evaluate only the results at the time t2 (Table 3). There was no statistical difference between bulls.

	t0	t1	t2	t3	SE
Conc(sp/ml)*	0.00^{A}	0.05^{A}	0.16^{B}	0.22^{B}	0.02
Motility (%)	0.00^{A}	36.73^{B}	39.81^{Ba}	26.27^{Bb}	4.06
^{A,B}Least square means in each column and line with no common superscript differ Significantly (P< 0.01). ^{a,b} Least square means in each column and line with no common superscript differ significantly (P< 0.05). *the concentration is million sperm/ml					

Table 1: Concentration of sperm (sp) and viability at each time point.

	0 volts	1 volts	5 volts	10 volts	SEM
Conc(sp/ml)*	0.00^{Aa}	0.07^{Ab}	0.18^{B}	0.19^{B}	0.02
Motility (%)	0.00^{A}	27.21^{Ba}	40.01^{B}	35.58^{Bb}	4.01
^{A,B} Least square means in each column and line with no common superscript differ significantly (P< 0.01). ^{a,b} Least square means in each column and line with no common superscript differ significantly (P< 0.05). *the concentration is million sperm/ml					

Table 2: Concentration of sperm and viability at different voltages.

Voltage	Sperm Concentration*	Motility (%)
0 volts	0.00 ± 0.07^{Aa}	0.00 ± 10.32^{A}
1 volts	0.11 ± 0.07^{abc}	40.73 ± 10.32^{B}
5 volts	0.22 ± 0.07^{b}	59.41 ± 10.32^{B}
10 volts	0.32 ± 0.07^{Bd}	63.41 ± 10.32^{B}
^{A,B} Least square means (± SEM) within each column without common superscripts differ significantly (P< 0.01).^{a,b} Least square means (± SEM) within each column without common superscripts differ significantly (P< 0.05),*the concentration is million sperm/ml		

Table 3: The effects of different voltages on sperm concentration and motility.

Experiment 2

There were no differences when we compared the electric channel (EC) to the Percoll discontinuous gradient on cleavage rate (t=0.133; df=8; sdev=11.9), embryo development rate (t=0.270; sdev=9.59; df=8) and comparison of percentage of embryos on cleaved (t=0.422; sdev=10.0; df =8) (Table 4).

Embryo sexing

A total of 110 blastocysts produced by the two systems were sexed (40 and 70 respectively for control and EC.). No bull effect on embryo sex distribution was demonstrated: the percentages of female embryos were 58.9% and 61%, (respectively for control and EC). These percentages follow the typical results obtained using this type of embryo culture medium [36].

Groups	COCs(n)	Cleaved(n)	Bl(n)	%Cleaved(sd)	%Bl(sd)	% Bl / Cleaved(sd)
Percoll	315	167	87	53.0 (9.91)	27.6 (9.86)	52.1(10.5)
EC	341	187	94	54.8(13.6)	27.6(9.32)	50.3(9.60)
^{A,B} Percentages with standard deviations (sd) within each column without common superscripts differ significantly (P< 0.01). ^{a,b} Percentages with standard deviations (sd) within each column without common superscripts differ significantly (P< 0.05)						

Table 4: Cleavage rate and embryo production using Percoll and EC device.

Discussion

Sperm selection is essential to obtain spermatozoa of good quality from frozen-thawed semen for in-vitro fertilization (IVF). Most spermatozoa are damaged during semen freezing and thawing processes. In the last decade, techniques were developed to try to take advantage of the sperm's electric charge. One simple method known as the Zeta test was developed using the electrokinetic potential (Zeta potential) of the sperm, which allows sperm to adhere to surfaces when they are suspended in solutions free of protein [39]. The Zeta test is a simple, inexpensive method requiring only a positively charged tube

and a sperm suspension free of protein. The authors demonstrated that the highest quality sperm were also the most electronegative [39]. While these authors showed the more electronegative sperm had improved morphology, hyperactivity and maturity they also showed that their motility was reduced [39]. Ainsworth et al. [6], using a more sophisticated model of electrophoretic separation, placed a sperm suspension in buffer in an electrophoresis chamber. The sperm were attracted to the cathode and using this method large numbers of negatively charged sperm could be collected in large quantities. The major problem with this method is the arduous cleaning that must be performed on the instrument compared to other sperm separation methods [40].

Based on the information gleaned from these previous electro-separation techniques we developed our methodology and the electric channel sperm separation device. Our results showed that we were able to separate high quality sperm after thawing the semen using the electro-separation chamber but in one-half time of the Percoll technique. No differences in between the fertilization rates were seen between the Percoll gradient and our EC separation system. The previous findings regarding the effects of Percoll, PVP and centrifugation [5,18] give even more value to our results, since with our new device we can replace the Percoll gradients entirely without reducing the embryo development rate. Our results are more important if we support them with the Simon's data [31], which shows that using the electric charge was possible to select the most low percentage of sperm DNA damage. Another benefit of our new device it is the processing time. Usually technicians need a lot of time to process the semen, from 40 minutes to 2 hours. This new technique reduces this time to 25 minutes and yields the same result as the other techniques. We were able to fertilize the same number of oocytes using sperm separated with this device that we were with discontinous gradients. The evaluation of sex was considered as an important parameter of embryo quality. No difference in sex ratio was seen between the EC and the discontinous gradients. The ability to reduce the timing of processing is frequently beneficial to in vitro embryo production procedures. Increasing the time that the gametes are out of the incubator at room temperature, can induce stress in the gametes. Thus reducing the timing of the procedure is likely to reduce gamete stress.

Conclusions

The EC is a quick, simple, low-cost (Table 5), and highly effective procedure that can be used to isolate bull sperm in frozen-thawed semen samples. The ability to enrich the sperm population with a high number of functional sperm makes this method a useful tool for animal and potentially human assisted reproductive technologies. This sperm selection method can be beneficial to improve IVF in cattle, and may also be applied to other domestic and farm animal species. However, further studies are required to determine whether the EC method could remove more DNA-damaged spermatozoa than other sperm separation techniques. In conclusion, the biophysical spermatozoa separation methods were effective for removal of non-motile spermatozoa, and this technique reduced the total semen processing time after thawing to 25 minutes.

Technique	Time (minutes)	Cost ($)
Discontinuous gradients		
Labor# (for procedure)	80	$39.20
Labor# (preparation)	30	$23.50
Medium**		$15.00
Percoll		$3.70
15 ml conical tubes (3)		$0.45
Total		**$81.85**
Swim Up		
Labor (for procedure)	60	$54.80
Labor (preparation)	20	$15.67
Medium		$7.00
BSA		$0.02
15 ml conical tubes (1)		$0.15
Total		**$77.64**
Electric Chamber (EC)*		
Labor (for procedure)	25	$19.58
Labor (preparation)	30	$23.50
Medium		$7.00

Device		$5.00
15 ml conical tubes (1)		$0.15
Total		**$55.23**

#Labor is calculated at $47/hour including fringe benefits and overhead costs.

All three techniques need a set of pipettes, a centrifuge and a sperm counting chamber(Burker, Neubauer, or other).*The sperm separation requires an electical power source (cost ranges from $500-1,000). Most labs already have one of these for electrophoresis.**The medium price is based on a commercially obtained medium and this cost may vary.

Table 5: Comparison of costs between the principal sperm selection techniques.

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Declaration of Interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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