The effects of magnetic separation on cryopreserved bovine spermatozoa motility, viability and cryo-capacitation status


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Summary

Cryopreservation is a technique used to preserve cells for long-time storage. It is widely used in agriculture to store male gametes in liquid nitrogen. The aim of this study was to determine the optimum thawing temperature and time for samples subjected to annexin V magnetic-activated cell sorting (AnMACS) as the sperm preparation technique. Pooled semen samples from three ejaculates were divided into two groups. The treatment group was subjected both to AnMACS and to being cryopreserved, whilst the control group was cryopreserved directly without MACS. Post-thaw analysis was carried out for samples thawed at either 20°C for 13 s, 37°C for 30 s, 40°C for 7 s, 60°C for 6 s or 80°C for 5 s. Sperm kinematics, viability and capacitation status were determined for samples subjected to all thawing temperatures described. Results showed that thawing at 37°C for 13 s for MACS-processed samples was a superior option compared with other thawing procedures; there was a significant difference in $P < 0.05$ values for curvilinear velocity (VCL μm/s) and sperm straightness (STR %) when samples were thawed at 40°C for 7 s, with fewer capacitated spermatozoa ($P < 0.05$) when samples were thawed at 37°C for 30 s, 40°C for 7 s or 60°C for 6 s. Hence, we can speculate that the use of AnMACS as the sperm preparation technique can somehow enhance sperm cryosurvival rate after cryopreservation, however the fertilization potential of these cells has yet to be determined.

Keywords: Capacitation, Magnetic-activated cell sorting system, Thawing, Viability

Introduction

Cryopreserved semen samples have been widely used in the breeding industry for artificial insemination (AI). This approach enables long-term storage of the semen before AI such that semen can be transported over long distances, quarantine of the semen can occur or samples can be used as a genetic bank to store commercially desirable traits. Advances in freezing techniques have improved the availability of superior genetic material, and various thawing procedures have also been employed to optimize maximum recovery of viable frozen spermatozoa at insemination (Córdova-Izquierdo et al., 2006; Aamdal & Andersen, 2007).

Thawing reverses the freezing process by forcing the cell back to its original path after various environmental changes (Anel et al., 2010). Other factors that contribute to the determination of suitable
thawing rates include extenders, freezing rate, glycerol concentration and the packaging method (Robbins et al., 1976; Li et al., 2006). According to the bifactorial theory of cryoinjury, ideal cell survival requires that the freezing rate and thawing rate be evaluated together (Mazur & Schmidt, 1968).

Various methods of thawing frozen bovine semen have been tried in order to achieve maximum recovery of viable spermatozoa. This approach includes experiments ranging from thawing cryopreserved semen in iced water (1–3 °C) to thawing cells at 75°C for a minimum of 30 s or 12 s respectively (Pickett et al., 1978; Pace et al., 1981). Studies by Pace et al. (1981) indicated that thawing at 37°C for a minimum of 30 s is an acceptable method for gaining superior post-thaw sperm quality and fertility in the field. In addition to the thawing procedure used, it is important to use a suitable semen preparation technique.

The most commonly used sperm preparation technique is sperm washing. It is believed that this technique is able to remove seminal plasma, and reduce the occurrence of free radicals through the process of replacing seminal plasma in cultured medium (Mortimer & Mortimer, 1990). At the present time, the sperm washing technique has been found to have unacceptable limitations. Its ability to separate damaged sperm from healthy sperm is questionable. A revision of the swim-up technique has been developed. This technique is based on the assumption that phosphatidylserine (PS) is expressed when spermatozoa enter an early phase of apoptosis. Identifying spermatozoa that express PS and excluding them from the semen is postulated to increase the likelihood of successful fertilization (Vermes et al., 1995). The system described here uses microbeads bound with annexin V, which is a 35–36 kDa phospholipid-binding protein. When PS is exposed, these microbeads bind to apoptotic spermatozoa that have membrane deterioration, and therefore can be easily detected and separated from other spermatozoa. This system is known as the magnetic-activated cell sorting (MACS) system (Grunewald et al., 2006). Previous studies have shown that MACS can yield motile, viable, morphologically normal spermatozoa that display higher cryosurvival rates with a higher fertilization potential in human sperm (Grunewald et al., 2001). Hence, this study had been replicated in animals to evaluate the effects of this technique on viability and capacitation status, especially in bovine spermatozoa.

According to Watson (1995), cryopreservation modifies sperm membranes to a partially capacitated state and makes them more reactive to the environment. Besides causing an apoptotic-like mechanism by the induction of PS translocation, the cryopreservation and thawing process induces many changes in sperm cells, including membrane disorder and cell death. Diminished motility and membrane changes, including sperm capacitation or acrosomal reaction, are some of the main forms of damage brought about by cryopreservation (Martin et al., 1996).

Past research has focused more towards the effects of AnMACS solely on human sperm, not on bovine sperm. Therefore, the focal points of this research were to study the effects of AnMACS on kinematics, viability and capacitation status of Bos taurus sperm when subjected to various thawing temperatures.

### Materials and methods

#### Semen collection

Fresh semen samples were collected from three adult Piedmontese breed bulls using an artificial vagina at the Institut Bioteknologi Veterinar Kebangsaan, (IBVK), Jerantut, Pahang, Malaysia. Three ejaculates were obtained; average yield per ejaculation was about 5 ml.

#### Semen preparation

The sperm suspension was divided into two separate fractions. The first fraction was subjected to AnMACS followed by cryopreservation and thawing, whereas the second fraction was cryopreserved without AnMACS and was designated the control group. Semen was separated by binding annexin V microbeads to PS, which was externalized on the sperm membrane, and using a MiniMacs kit (Miltenyi Biotec, GmbH, Germany). Semen were centrifuged for 10 min at 300 relative centrifugal force (rcf). Supernatant was removed completely and the cell pellet was resuspended in 80 μl of annexin V buffer per 10^7 cells. Next, 20 μl of MACS annexin V microbeads were added per 10^7 total cells. The mixture was incubated for 15 min in the dark at 10°C. The sperm were then washed with 1–2 ml buffer per 10^7 cells and resuspend in 500 μl buffer per 10^8 total cells. The cells were added to the separation column, which was placed in a magnet such that the annexin V microbead-labelled spermatozoa (ANMB-positive) were retained in the separation column. The fraction with intact membranes that passed through the column was labelled as the ANMB-negative fraction. This fraction was eluted and collected from the column (Said et al., 2005; Said et al., 2008).

#### Cryopreservation-thawing protocol

The control group and ANMB-negative fraction was cryopreserved using Bioxcell® extender (IMV, France). The concentration of semen mixture was determined using a SpermaCue photometer (Miltenyi Biotec Asia Pacific Pte Ltd, Singapore) and adjusted to
20 million spermatozoa per 0.25 ml straw. The mixture was placed in chiller at 4°C for at least 3 h. Samples were then transferred to 0.25 ml straws and put in liquid nitrogen vapour (–70°C) for 9 min. Finally, the straws were plunged into liquid nitrogen (–196°C) and stored in liquid nitrogen tanks. The direct plunging cryopreservation technique was used.

Straws were then thawed in water baths at 20°C for 13 s, 37°C for 30 s, 40°C for 7 s, 60°C for 6 s and 80°C for 5 s respectively.

Eosin–nigrosin staining technique
The viability of spermatozoa was assessed by the eosin–nigrosin staining method. For eosin–nigrosin staining, the final composition of the stain was eosin-Y (1.67 g), nigrosin (10 g), and sodium citrate (2.9 g), dissolved in 100 ml distilled water. Sperm suspension smears were prepared by mixing a drop of sperm sample with two drops of stain on a warm slide and spreading the stain with a second slide. The smear was allowed to dry. Viability was assessed by counting 200 spermatozoa under a bright-field microscope at ×400 magnification. Sperm that displayed partial or complete purple staining were considered to be non-viable whilst only sperm that showed strict exclusion of stain were counted as viable (Kim et al., 2009). Images were captured using phase-contrast inverted microscope with an analyzer system (Olympus 1 × 51, and Image Pro Plus 5.1 software).

Chlorotetracycline staining assay
A modified chlorotetracycline (CTC) staining protocol was used to determine the sperm cell capacitation status (Ward & Storey, 1984). Hundreds of 50 ml of washed sperm were mixed with 3 ml propidium iodide (PI) and 3 ml Hoechst342 stain (40 μg/ml) and incubated for 15 min at 37°C in the dark. One drop of the mixture was added to one drop of CTC solution (805 μmol/l CTC and 5 mmol/l cysteine in CTC buffer that contained 130 mmol/l NaCl and 20 mmol/l Tris (Trizma base, pH 7.8) and fixed immediately with one drop of 12.5% (w/v) paraformaldehyde in 1 mol/l Tris–HCl, pH 8.0. Next, a drop of 0.22 M 1,4-diazabicyclo[2.2.2]octane (DABCO) was added to the mixture to prevent fading of fluorescence. Two slides were prepared per treatment samples and analyzed within 2 h of preparation.

Chlorotetracycline staining was assessed on live spermatozoa under blue-violet illumination (excitation at 330–380 nm, emission at 420 nm). The staining patterns observed was similar to those described by Wang et al. (1995), namely: F, full fluorescence (uncapacitated); B, a fluorescence-free band in the post-acrosomal domain (capacitated); and AR, low fluorescence over the entire head with a band of bright fluorescence across the equatorial segment (acrosome reacted cells).

Analysis of sperm parameters
Sperm count and motility were performed using a CEROS sperm analyser (Hamilton Thorne Inc, Beverly, MA, USA). Five straws were thawed per treatment in triplicate and analyzed for computer-assisted sperm analysis (CASA) parameters of sperm velocity and sperm progression. Briefly, 10 μl of fresh semen were diluted at a 1:9 ratio in 3% sodium citrate to access CASA parameters.

For sperm velocity, three parameters were considered, these were average path velocity (VAP) μm/s, straight line velocity (VSL) μm/s and curvilinear velocity (VCL) μm/s. Sperm velocity is a measure of how fast sperm are moving along their trajectory. Curvilinear velocity is defined as the time-average velocity of a sperm head along its actual curvilinear path. Straight line velocity is the average time-velocity of a sperm head in a straight line between its first and last spotted point. Average path velocity is the time-average velocity of sperm along its average path (Ibrahim et al., 2011). Sperm progression is the movement pattern along its path. Wobble (WOB %), describes the sperm head movement side to side, linearity (LIN %) explains the trajectory straightness of the sperm’s path and STR (%) illustrates the average distance of the sperm from its origin on the average path in all frames (Ibrahim et al., 2008).

Statistical analysis
Two-way analysis of variance (ANOVA) was used to determine the effects of the thawing procedures on the control and treatment groups assessed for sperm kinematics, viability and capacitation status. Results were reported as mean ± standard error of the mean (SEM). A P-value of 0.05 was considered to be significant.

Results
Following the cryopreservation–thawing process, AnMACS positively selected sperm differed significantly only in velocity (VCL μm/s) and sperm straightness (STR %), which displayed significantly higher values compared with the control group at a P-value <0.05 (Table 1). The effect of AnMACS on sperm was mostly apparent on sperm velocity, especially when samples were thawed at 37°C for 30 s. For sperm progression the effect of MACS was significant when cells were thawed at 40°C for 7 s or at 60°C for 6 s, P < 0.05.

Furthermore, in sperm progression, two-way ANOVA showed that WOB (%), LIN (%) and STR (%) were all affected by inclusion in control or treatment
Table 1 Motility characteristics (mean ± SEM) for sperm velocity, progression and viability in thawed bull semen after magnetic separation (n = 200).

<table>
<thead>
<tr>
<th>Thawing temperature (°C)</th>
<th>VAP (μm/s)</th>
<th>STR (%)</th>
<th>Lt</th>
<th>WOB (%)</th>
<th>LIN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 20/13</td>
<td>79.10 ± 1.92</td>
<td>81.55 ± 2.38</td>
<td>1.88</td>
<td>18.78 ± 2.43</td>
<td>74.43 ± 4.08</td>
</tr>
<tr>
<td>Treatment 20/13</td>
<td>81.88 ± 2.19</td>
<td>87.52 ± 1.97</td>
<td>1.99</td>
<td>15.18 ± 2.04</td>
<td>74.36 ± 3.08</td>
</tr>
<tr>
<td>Treatment 20/13</td>
<td>89.30 ± 2.01</td>
<td>91.65 ± 2.92</td>
<td>2.03</td>
<td>12.39 ± 2.49</td>
<td>74.25 ± 2.81</td>
</tr>
<tr>
<td>Treatment 20/13</td>
<td>90.65 ± 2.35</td>
<td>91.65 ± 2.92</td>
<td>2.03</td>
<td>12.39 ± 2.49</td>
<td>74.25 ± 2.81</td>
</tr>
<tr>
<td>Control 37/30</td>
<td>87.73 ± 2.46</td>
<td>87.44 ± 6.07</td>
<td>1.59</td>
<td>72.85 ± 7.22</td>
<td>81.13 ± 0.74</td>
</tr>
<tr>
<td>Treatment 37/30</td>
<td>89.88 ± 2.01</td>
<td>91.65 ± 2.92</td>
<td>2.03</td>
<td>12.39 ± 2.49</td>
<td>74.25 ± 2.81</td>
</tr>
<tr>
<td>Treatment 37/30</td>
<td>90.65 ± 2.35</td>
<td>91.65 ± 2.92</td>
<td>2.03</td>
<td>12.39 ± 2.49</td>
<td>74.25 ± 2.81</td>
</tr>
<tr>
<td>Control 40/7</td>
<td>86.52 ± 3.01</td>
<td>81.55 ± 2.38</td>
<td>1.88</td>
<td>18.78 ± 2.43</td>
<td>74.43 ± 4.08</td>
</tr>
<tr>
<td>Treatment 40/7</td>
<td>89.30 ± 2.01</td>
<td>91.65 ± 2.92</td>
<td>2.03</td>
<td>12.39 ± 2.49</td>
<td>74.25 ± 2.81</td>
</tr>
<tr>
<td>Treatment 40/7</td>
<td>90.65 ± 2.35</td>
<td>91.65 ± 2.92</td>
<td>2.03</td>
<td>12.39 ± 2.49</td>
<td>74.25 ± 2.81</td>
</tr>
<tr>
<td>Control 60/6</td>
<td>79.37 ± 1.87</td>
<td>80.52 ± 2.38</td>
<td>1.99</td>
<td>15.18 ± 2.04</td>
<td>74.36 ± 3.08</td>
</tr>
<tr>
<td>Treatment 60/6</td>
<td>89.30 ± 2.01</td>
<td>91.65 ± 2.92</td>
<td>2.03</td>
<td>12.39 ± 2.49</td>
<td>74.25 ± 2.81</td>
</tr>
<tr>
<td>Treatment 60/6</td>
<td>90.65 ± 2.35</td>
<td>91.65 ± 2.92</td>
<td>2.03</td>
<td>12.39 ± 2.49</td>
<td>74.25 ± 2.81</td>
</tr>
<tr>
<td>Control 80/5</td>
<td>85.57 ± 1.92</td>
<td>80.52 ± 2.38</td>
<td>1.99</td>
<td>15.18 ± 2.04</td>
<td>74.36 ± 3.08</td>
</tr>
<tr>
<td>Treatment 80/5</td>
<td>89.30 ± 2.01</td>
<td>91.65 ± 2.92</td>
<td>2.03</td>
<td>12.39 ± 2.49</td>
<td>74.25 ± 2.81</td>
</tr>
<tr>
<td>Treatment 80/5</td>
<td>90.65 ± 2.35</td>
<td>91.65 ± 2.92</td>
<td>2.03</td>
<td>12.39 ± 2.49</td>
<td>74.25 ± 2.81</td>
</tr>
</tbody>
</table>

The study was done in triplicate.

The study significance level was set at P < 0.05 between control and treatment groups, SEM, standard error of the mean. LIN, linearity; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, wobble.

Spermatozoa viability assessment through eosin–nigrosin staining technique

The commonly used method for spermatozoa viability is eosin–nigrosin staining. Eosin is an orangish pink to red dye stains the cytoplasmic material. It penetrates in non-viable cells, which then appear red. Nigrosin gives a dark background, facilitating the detection of viable, non-stained cells (Figs. 1 and 2).

Statistical analysis revealed that thawing at 20°C for 13 s, 37°C for 30 s or 40°C for 7 s yielded significantly lower values for sperm viability P = 0.005, P = 0.02 and P = 0.03 respectively compared with the control group (Table 1).

Cyro-capacitation status

After thawing at 37°C for 30 s, 40°C for 7 s or 60°C for 6 s, there was a significantly higher number of sperm in an uncapacitated state in the MACS-treated sample. Meanwhile, at the same temperatures, fewer capacitated sperms were found in the MACS-treated sample compared with the control group, P < 0.05.

Discussion

At present, one of the main important parameters used to evaluate semen quality is sperm motility. CASA software is one option used to represent motility and viability of sperm. Parameters of sperm motility have been divided into two categories, namely sperm velocity (VCL, VSL, VAP; μm/s) and sperm progression (WOB, LIN, STR; %). The thawing temperatures and time selected for this study were based on results from previous studies (Nur et al., 2003; Muiño et al., 2008; Calamera et al., 2010). Methodologies were combined and the best thawing group, thawing temperature and time (P < 0.05). Only STR (%) in the treatment group showed a significantly higher value compared with the control group when cells were thawed at 40°C for 7 s or 60°C for 6 s.

In the AnMACS-treated group, sperm viability was mostly reduced compared with the control group. The highest per cent viability (43.2 ± 2.89%) was found in the control group following thawing at 40°C for 7 s. Nevertheless, the CTC staining pattern revealed that AnMACS-treated sperms showed a significantly higher number of uncapacitated sperms compared with the control group after thawing 37°C for 30 s, 40°C for 7 s or 60°C for 6 s.

Most characteristics of sperm motility were not changed, except for VCL (μm/s) and STR (%), which were higher in the treatment group. Hence, the reliability of this system was shown only for sperm VCL (μm/s), STR (%) and uncapacitated sperm.
temperatures and times were chosen to suit the goals for our study.
In this study, the most significant changes on sperm motility could be seen for the AnMACS-treated samples, for VCL (μm/s) and STR (%). According to Muiño et al. (2008) spermatozoa with relatively low velocity (medium VCL and VAP) and high progressiveness (high LIN %, STR % and WOB %) could be classified as subpopulation 1, comprised of relatively slow but progressive spermatozoa. This slow progression was a result of post-thaw thermal stress, as some of the spermatozoa may reduce their flagella activity, probably due to sublethal cryoinjury, although their movement is still forward. This type of subpopulation was observed when thawing was carried out at 80°C for both control and treatment groups. Furthermore, spermatozoa that exhibited highly active but non-progressive movement with high values of VCL μm/s, low LIN % and low STR % were observed when thawing was performed at 20°C for 13 s. These spermatozoa can be classified as subpopulation 2 and could be considered as having ‘hyperactivated-like’ movement. This pattern of movement is due to the accumulation of intracellular Ca²⁺ above physiological concentrations (Yanagimachi, 1994).
Our study indicated that sperm move most rapidly and progressively after AnMACS and when thawed at 37°C for 30 s, presumably at this temperature energy production and cell respiration occur at their maximum levels. As we can see from the results, there is a crossover of results at certain temperatures between control and treatment samples. For the average path velocity (VAP μm/s) the crossover was at 70°C, whilst for curve line velocity (VCL μm/s) the intercepts were at 37°C and 70°C. This phenomenon might be due to spermatozoa changes in VAP μm/s and VCL μm/s at these temperatures, especially for AnMACS-treated spermatozoa. After incubation at 70°C, separated spermatozoa that have undergone AnMACS are not tolerant to heat stress and hence decrease their velocity. However, different phenomena occurred for progression parameters (WOB %, LIN % and STR %). Results obtained indicated that spermatozoa progression was increased as the temperature increased and that progression was increased by decreasing velocity. These types of motility, with low velocity and high progression, are not suitable for sperm to achieve fertilization with the egg. Hence, a compromise between velocity and progression is needed in order for the sperm to successfully penetrate the egg.
Survival of the spermatozoa after the freezing–thawing process depends on the plasma membrane, as this is the most crucial region and regarded as the primary site of cryoinjury (Söderquist et al., 1997). The AnMACS preparation technique was believed to yield motile, viable, morphologically normal spermatozoa that displayed higher cryosurvival rates as well as higher fertilization potential (Grunewald et al., 2001; Said et al., 2005). Moreover, non-apoptotic sperm separated by AnMACS prior to cryopreservation had significantly higher motility following cryopreservation.
thawing than sperm that were not separated by MACS (Said et al., 2008).

Furthermore, it is believed that membranes are more intact in the AnMACS-processed samples as there is no phosphatidylserine to be exposed. AnMACS is an excellent method for separating cells of interest out of a mixed cell population. The principle underlying this tool is the utilization of magnetic micro-particles conjugated with specific antibodies. Untagged cells are not influenced by the magnetic field and can be used directly for insemination (Kang & Park, 2004). Additionally, AnMACS usage allows the enrichment of cells with and without PS exposure on their surface. Externalization of plasma membrane PS is part of an oxidative signalling pathway, identified as one of the early and prominent features of apoptosis. In cells, PS exposure on the outer leaflet on the plasma membrane has several functions, one of which is recognition and removal of apoptotic cells by phagocytes (Kagan et al., 2000). In the present study, sperm were labelled magnetically with annexin V microbeads and passed through a MACS column, which was placed in the magnetic field of a MACS separator. Cells with externalized PS were tagged, separated from the normal healthy cells and then flushed out from the system. As a result, only healthy viable cells were collected for cryopreservation. Removal of abnormal spermatozoa and spermatozoa undergoing apoptosis can decrease the oxidative stress among vital healthy sperm (Dirican et al., 2008).

The results found in this study might relate to decreased oxidative stress in the ejaculate after magnetic separation. In other words, AnMACS acts on sperm at the molecular level and this technique can be considered as an effective method in sperm preparation to enhance sperm quality and function. AnMACS is also a simple, reliable and rapid sorting system for separating sperms in large numbers (Said et al., 2005).

It is clear that fertility is reduced in half the number of sperm that have undergone the cryopreservation process compared with fresh semen. It is now recognized that the surviving population of spermatozoa is compromised as a result of capacitation-like changes (Watson, 1995; Bailey et al., 2000). In this study, the capacitation status of AnMACS-treated spermatozoa and control spermatozoa were assessed using the CTC assay. Results indicated that AnMACS-treated samples produced more uncapacitated spermatozoa compared with the control group (Figs. 3 and 4), even after
different thawing procedures were applied. Kurz et al. (2005) reported that PS is localized in the cytoplasmic leaflet of the plasma membrane as well as on the outer acrosomal membrane. During the capacitation and acrosome reaction, PS was not exposed on the surface of the viable sperm cells. This point clarifies and adds weight to our result that more uncapacitated cells were revealed during the capacitation and acrosome reaction as a consequence of magnetic separation. This point furthermore explains the fact that good quality sperms did not expose PS on the outer leaflet even though they were undergoing an extreme event such as cryopreservation, which is believed to trigger cryo-capacitation-like damage.

Results further showed that thawing at 37°C, 40°C or 60°C respectively yielded significant results for AnMACS-processed samples rather than for the control group (P < 0.05). Here we postulated that using AnMACS as the sperm preparation technique and thawing at either one of these three temperatures will yield spermatozoa that are yet to be capacitated. This factor is because capacitation must occur at the exact time it is inside the female oviductal tract in order for fertilization to take place. Any events preceding fertilization that occur at the wrong time and/or the wrong place will result in poor fertilization rates (Donnelly et al., 1998). This cryo-capacitation mechanism, because of the onset of normal capacitation, has yet to be determined. It has been reported that reactive oxygen species (ROS) have a significant physiological role during normal sperm function, including hyperactivation, capacitation and acrosome reaction (de Lamirande et al., 1997), and ROS is also believed to play an important function in fertility or infertility. Furthermore, according to Langlais and Roberts (1985) capacitation of mammalian sperm occurs when the head of plasma membrane undergoes reorganization due to phospholipids redistribution and cholesterol removal. This membrane destabilization causes the sperm to exhibit cryo-capacitation with a B pattern that favours calcium influx to the cell during cryopreservation (Maxwell & Johnson, 1997). Moreover, the capacitation process is believed to be related to apoptosis. ROS are essential in regulating sperm function, but ROS also act as a two-edge sword (Aitken, 2011). At low levels ROS are needed to promote cholesterol oxidation and tyrosine phosphorylation events that support capacitation. Meanwhile, excessive production of ROS can lead to a state of oxidative stress that affects sperm function. Oxysterols, the oxidized derivatives of cholesterol, act as a good indicator of oxidative stress but might also be efficient inducers of this process. Accumulation of oxysterols has been linked to the activation of apoptosis and cell death in other cell types (Ryan et al., 2005).

Moreover, mitochondria appear to be key mediators of oxysterol-induced pathology by means of the intrinsic apoptosis pathway. One type of ROS is peroxynitrite, which functions in stimulating cholesterol oxidation and oxysterol formation. Peroxynitrite is known to be the promoter for capacitation in several mammalian species (Rodriguez et al., 2011). Besides this function, it also activates oxysterol formation in cells. Previously, Brouwers et al. (2011) found that oxysterol generation is disrupted after cryopreservation, whereas lipid peroxidation is generally enhanced. Elevated oxysterol formation in mitochondria could lead to activation of an intrinsic apoptotic cascade and begin a number of cellular changes such as sperm senescence, enhanced mitochondrial superoxide generation, motility loss, caspase activation and PS exposure. Therefore, we can state here that the AnMACS procedure selects sperms with less oxidative stress, based on externalization of PS, hence the outcome will be less capacitated spermatozoa.

During this experiment, eosin–nigrosin stain was used to measure sperm viability. Spermatozoa that stained red or pinkish were considered to be non-viable. Sperm take up the dye when the integrity of the membrane is badly damaged, indicating that spermatozoa physiological function was not operating well. Even though per cent viability values were only significant (P < 0.05) between control and treatment groups when sperm were thawed at 20°C for 13 s or 40°C for 7 s, samples that had undergone AnMACS prior to cryopreservation could preserve their viability up to a temperature as high as 60°C compared with the control group, which could only survive up to 40°C. This finding may indicate that magnetic separation based on the externalization of PS had successfully yielded a sperm subpopulation that was heat resistant. Furthermore, from results obtained, AnMACS-treated sperms had increased viability when the temperature increased from 20°C to 60°C. Although the viability of MACS-processed sperms was less compared with sperm in the control group at 20°C and 40°C, viable sperm with less PS were selected. Sperm without this system might be viable but be facing apoptosis; hence fertilization outcome might not be good if we continue to select this subpopulation.

Conclusion

In conclusion, MACS might act as an ideal sperm preparation technique prior to cryopreservation. Use of AnMACS produced sperm with higher VCL μm/s and STR % after thawing at 37°C for 30 s or 40°C 7 s respectively. This pattern indicates that sperm were exhibiting faster and straighter movement, which may greatly affect in vitro fertilization (IVF) success rates.
In addition, by using AnMACS in sperm preparation, we are able to select the most viable spermatozoa for cryopreservation. MACS-processes samples can sustain their viability up to higher temperatures as much as 60°C. Furthermore, another significant finding that emerged from this study was that less capacitated spermatozoa were likely to be present after thawing at 37°C for 30 s, 40°C for 7 s or 60°C for 6 s in the AnMACS-treated samples. Hence, the recommended thawing temperature can be at 37°C for 30 s, 40°C for 7 s or 60°C for 6 s. But, during this study a thawing temperature at 37°C for 30 s was chosen because of the ease in temperature manipulation in field work. However, the fertility potential of this AnMACS sperm population has yet to be determined. This result could be achieved through further future research.

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