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Quantification of HSP70 Gene Expression and Determination of Capacitation Status of Magnetically Separated Cryopreserved Bovine Spermatozoa at Different Thawing Temperature and Time

(Kuantifikasi Ekspresi Gen HSP70 dan Penentuan Status Kapasitasi Sperma Lembu yang telah Dikrioawet pada Suhu Pencairan dan Masa yang Berbeza)

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ABSTRACT

The role of heat shock protein in reproduction is widely known as a molecular chaperone in aiding and repairing protein formation when stress occurred. The present objectives were to evaluate the effect of different thawing temperature and time on the expression of HSP70 gene expression and the capacitation status in cryopreserved bovine spermatozoa. Briefly, fresh ejaculates were obtained from three different adult bulls. The semen then underwent a sperm washing technique known as Magnetic Activated Cell Sorting System (MACS) and later on, cryopreserved. The sperm- containing straws were then thawed at five different thawing temperatures and time post-cryostorage; 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. The RNA was extracted from each of the sperm's pellets and converted to cDNA prior to the qPCR process. Capacitation status was then determined by means of CTC assay. The results showed that after the process of amplification, there is a significant different of HSP70 gene expression in MACS process samples when the thawing process was performed at 37°C for 30 s, with $p<0.05$. *Furthermore, the CTC assay also showed that thawing at the same temperature gave less capacitated spermatozoa with* $p<0.05$. *As a conclusion, MACS yield spermatozoa with a better expression of HSP70 gene and less capacitated spermatozoa when thawing was done at 37°C for 30 s.*

Keywords: CTC; heat-shock protein; MAC; sperm cryopreservation; thawing

ABSTRAK

Peranan protein kejutan haba dalam pembiakan telah diketahui secara meluas terutamanya sebagai molekul pengiring dalam membantu dan memperbaik pembentukan protein apabila tekanan berlaku. Objektif semasa adalah untuk menilai kesan suhu dan masa pencairan yang berbeza pada ekspresi gen protein HSP70 dan status kapasitasi sperma lembu yang telah dikrioawet. Secara ringkas, ejakulasi segar diperoleh daripada tiga lembu jantan dewasa yang berbeza. Air mani kemudiannya menjalani teknik penyediaan sperma yang dikenali sebagai Magnetic Activated Cell Sorting System *(MACS) dan kemudiannya dikrioawet. Selepas itu, straw yang mengandungi sperma kemudiannya dicairkan pada lima suhu pencairan dan masa yang berbeza; 20°C selama 13 s, 37°C selama 30 s, 40°C selama 7 s, 60°C selama 6 s dan 80°C selama 5 s. RNA kemudian diekstrak daripada pelet sperma dan ditukarkan kepada cDNA sebelum proses qPCR. Status kapasitasi kemudiannya telah dilakukan melalui esei CTC. Keputusan kajian menunjukkan bahawa selepas proses penguatan, ekspresi gen HSP70 terdapat perbezaan yang signifikan terhadap sperma yang telah menjalani MACS apabila pencairan dilakukan pada suhu 37°C selama 30 s dengan nilai* $p<0.05$. *Tambahan pula, asai CTC juga menunjukkan bahawa pencairan pada suhu yang sama memberikan sperma yang kurang berkapasiti dengan nilai* $p<0.05$. *Kesimpulannya, MACS boleh memberikan spermatozoa yang berkualiti baik dengan ekspresi HSP70 yang tinggi dan sperma yang kurang berkapasiti apabila pencairan dilakukan pada 37°C selama 30 s.*

Kata kunci: CTC; krioawet sperma; MACS; pencairan; protein kejutan haba

INTRODUCTION

Cryopreservation of semen is successfully used widely in the agricultural world due to the advantages it promises. Cryopreservation allows genetic improvement of agriculturally important species as well as controlling the spread of sexually transmitted disease. These factors are fundamentally important to succeed a sustainable agri-food industry (Bailey et al. 2000). From this method, farmers can now particularly select the semen from bulls that might not be available when required. Using the Assisted Reproductive Technique (ART) especially Artificial Insemination (AI), only one ejaculate from a genetically superior male can be used to impregnate multiple females in order to maximize the distribution of important and favorable genes. However, the process of thawing exerted some sort of stress towards these spermatozoa by impairing its motility and viability. For that reason, various kind of proteins is released in order to

repair those damaged proteins. These proteins are known as the Heat-Shock Protein (HSP). These heat-shock proteins can be divided into different families which are based on their molecular weight measured in kDa rather than by their function. There are 27, 60, 70 and 90 kDa HSP which is in small families. In reproduction, HSP60 and HSP70 are most important HSP, serving two major functions as a molecular chaperone and responding to cellular stress such as changes in temperature, free oxygen radical, viral and bacterial infection as well as heavy metals (Neuer et al. 2000). Studies have verified that HSP, particularly the most abundantly expressed 70 kDa HSP, plays an important roles in acquiring thermal tolerance as an indicator (Huang et al. 2000; O'Hara et al. 2010). After ejaculation, semen is processed and prepared in order to obtained good quality sperm. The current standard of sperm preparations are the migration and sedimentation concept which is based on the sperm motility or density solely. However, the molecular event such as apoptosis is ignored, which may negatively impacts the fertility potential of the spermatozoa (Said et al. 2008). Therefore, the latest sperm separation technique called magnetic activated cell sorting system (MACS) was developed to avoid such impact. This system uses Annexin V microbeads which is a 35-36 kDa phospholipid binding protein. When phosphatidylserine (PS) was exposed, these microbeads will bind to the apoptotic spermatozoa with deteriorated membranes so that it can be easily detected and separated from other spermatozoa. The objectives of this study were to quantify the expression of heat-shock protein (HSP70) gene and determine the capacitation status of spermatozoa after subjected to MACS and various thawing temperature and time.

Materials and Methods

Semen Collection

Fresh semen samples were collected from three adult bulls (Piedmontese) using artificial vagina at Institut Bioteknologi Veterinar Kebangsaan, (IBVK), Jerantut, Pahang. Three ejaculates were obtained and 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 magnetic activated cell sorting (MACS) system (MiltenyiBiotec, GmbH, Germany) mini macs kit followed by cryopreservation and thawing process, whereas the second fraction was cryopreserved without subjected to MACS system as the control group. The semen from the first fraction was separated based on the binding of Annexin V microbeads to phosphatidylserine that was externalized at the sperm's membrane. Briefly, the semen was subjected to centrifugation for 10 min at 300 rcf. The supernatant was then removed completely and the cell pellet was re-suspended in 80 µL of Annexin V buffer per 10^7 cells. 20 µL of MACS Annexin V microbeads were added and the mixture was then incubated for 15 min in the dark at 6 -12°C. The sperms were then washed once with 1.5 mL buffer and re-suspended in 500 µL of the same buffer. Cells were then placed on top of the separation column containing iron balls. The Annexin V microbeads-labelled spermatozoa (ANMB-positive) were retained in the separation column, which was placed in a magnet. The fraction with intact membranes that passed through the column was labeled as (ANMB-negative) fraction. This fraction was eluted and collected from the column. The magnetic field power used was 0.5 Tesla between the poles of the magnet and up to 1.5 Tesla within the iron globes of the column (Said et al. 2005).

Semen Cryopreservation and Thawing Protocols

The second fraction (control group) and ANMB negative fraction were cryopreserved using Bioxcell® extender (IMV, France). The concentration of semen mixture was determined using the (SpermaQue, German) and adjusted to 20 million spermatozoa per 0.25 mL straw. The mixture was placed in a chiller at 4°C for at least 3 h. The sample was then transferred to 0.25 mL straws and put onto the vapor of liquid nitrogen (-70°C) for 9 min. Finally, the straws were then plunged into the liquid nitrogen (at -196°C) and stored in liquid nitrogen tanks.

Straws were then thawed in a water bath 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. Thawing regimes were chosen based on previous studies and were slightly modified. These studies stated that various thawing rates would exert different effects towards sperms survival (Al-Badry 2012; Calamera et al. 2010; Rastegarnia et al. 2013). The temperatures chosen were ranged from as low as 20°C until 80°C.

Extraction of Total RNA and cDNA Synthesis

Conventional mortar and pestle technique combined with RNA extraction kit (Norgen Biotek, Canada) was used for RNA extraction. Briefly, 20 straws were thawed and centrifuged at 200 rcf for 15 min in order to collect the pellet. The pellet was transferred into mortar and pestle with an appropriate amount of liquid nitrogen to cover up the sample. The sample was then grounded into a fine powder using pestle in liquid nitrogen. Liquid nitrogen was allowed to evaporate without allowing the sample to thaw. The next steps were performed under manufacturer instructions. The concentrations of extracted RNA were determined by using Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies Inc, USA). The RNA was converted to cDNA before preceded into PCR by using iScript cDNA synthesis kit (Biorad, CA). This step was performed by adding 4 µL of 5X I Script buffer, 1 µL iScript reverse transcriptase, 10 µL RNA template and nuclease free water was added to total volume of 20 µL. The reaction protocol was carried out at 25°C for 5 min, 42°C for 30 min, 85°C for 5 min and was held at 4°C until taken to storage. Synthesized cDNA was stored at -20°C until further use.

REAL-TIME POLYMERASE CHAIN REACTION (qPCR)

The primers were designed using Primer3 software and BLAST at NCBI webpage. The primers were obtained according to the sequence of Bos taurus 70 kDa heat-shock protein (hsp70) mRNA (forward), 5' CCTCGTACACCTGGATCAGC- 3' and (reverse), 5'- CTGATGGGGGACAAGTCG - 3'. As for housekeeping gene, Bos taurus glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA, (forward) 5'-CCACCACCCTGTTGCTGTAG-3, (reverse) 5'-CTGAGGACCAGGTTGTCTCC-3'. The reaction was performed in 25 µL volumes using 12.5 µL of IQ SYBR Green (Biorad, CA), 1.25 µL of each forward and reversed primer (1st Base, Malaysia), 2 µL of cDNA and 8 µL molecular biology grade water. The reaction condition was as follows: Enzyme activation at 95°C for 30 s, 95°C for 3 min, 49 cycles of denaturation at 95°C for 10 s, annealing/extension at 61.4°C for 30 s and melt curve from 55°C - 95°C (in 0.5°C increment) for 10 s/step. The reaction took about 2 h and 30 min. In qPCR, the chosen primer has an expected product size of 196 base pair. The Ct values were obtained from amplification chart while the melting curve was performed in order to check if the amplified fragments were correctly performed by applying the formula of gene expression = $2^{(\text{Ct value housekeeping gene} - \text{Ct value gene of interest})}$.

CHLORTETRACYCLINE (CTC) ASSAY

Cryopreservation most likely will exert damages toward sperm's membrane integrity. One of the method used to assess cryo-capacitation like damage cause by cryopreservation is the Chlorotetracycline/Hoechst Staining Assay (CTC). Modified chlortetracycline staining protocol was used to determine the sperm cell capacitation status (Oh et al. 2010). Hundreds of fifty microliters of washed sperm were mixed with 3 µL Propidium Iodide (PI) and three micro litres Hoechst342 (40 µg/mL) and incubated for 15 min at 37°C in the dark. 10 µL of the mixture was added with 10 µL of CTC solution (805 µmol/l chlortetracycline and 5 mmol/l cysteine in chlortetracycline buffer containing 130 mmol/l NaCl and 20 mmol/l Tris (Trizma base, pH7.8) and fixed immediately with 10 µL of 12.5% (w/v) paraformaldehyde in 1 mol/l Tris–HCl, pH8.0. Then, the mixture was added with a droplet of 0.22 mol 1,4-diaza bicyclo [2.2.2] octane (DABCO) to prevent the fading of fluorescence stain. 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). 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).

STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS software, version 14.0. The significant different of results between thawing procedures for sperm HSP70 gene expression and capacitation status for control and treatment groups were determined using two-way ANOVA. The p-value of 0.05 was considered as significant.

RESULTS

RNA EXTRACTIONS FROM BULL SPERMS

RNA bands were observed following gel electrophoresis (0.5% w/v) from control and treatment group. The smearing band may indicate that the RNA had undergone degradation process (Figure 1).

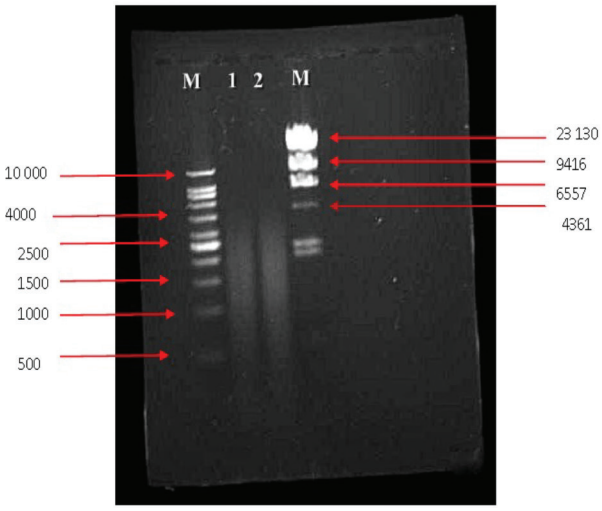


FIGURE 1. Analysis of extracted bull sperms RNA on a 0.5% agarose gel. Lane from extremely left: Lane 1: 1 Kb DNA ladder, Lane 2: RNA sample 1 (control), Lane 3: RNA sample 2 (treatment), Lane 4: Lambda *Hin*dIII marker

RNA concentration of treatment group was the highest following cryopreservation and thawing process that was done at 37°C for 30 s with 83.84 ng/µL. The high value of RNA purity indicates that the RNA was free from any contamination and excess ethanol (Table 1).

DNA AMPLIFICATION USING REAL-TIME PCR *(qPCR)*

ANOVA test performed, a significant value was observed of the HSP70 gene expression when thawing was done at 37°C for 30 s and 60°C for 6 s with $p<0.05$ (Table 2). Thawing temperature of 30°C for 30 s showed the highest value of HSP70 gene expression in MACS treatment group.

qPCR PRODUCT CONFIRMATION ON 1.5% AGAROSE GEL

After qPCR was carried out, the products were validated and confirmed on 1.5% (w/v) of agarose gel electrophoresis under UV trans illuminator (Figure 2). The result indicated that all PCR products were successfully amplified with an expected base pair of 196.

CRYO-CAPACITATION LIKE DAMAGE BY CHLOROTETRACYCLINE/HOECHST STAINING ASSAY

The result showed that the thawing temperature of 37°C for 30 s, 40°C for 7 s and 60°C for 6 s were significantly affects uncapacitated and capacitated sperms with $p<0.05$ (Table 3).

TABLE 1. Optical density (OD) readings for extracted RNA in control and treatment group

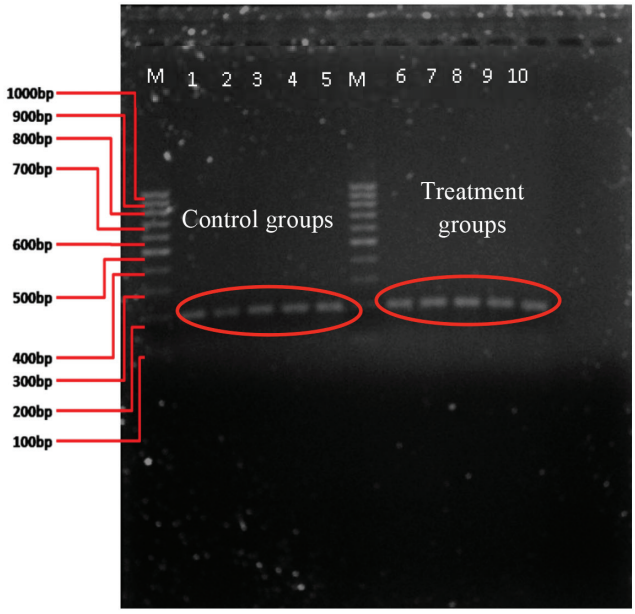
Sample id	ng/µl	A_{260}	$A_{260/230}$	$A_{260/280}$	Constant
RNA fresh sample	12.68	0.317	0.38	1.56	40
RNA 20°C 13 s (control)	27.05	0.676	0.46	1.78	40
RNA 20°C 13 s (treatment)	33.47	1.272	0.64	1.82	40
RNA 37°C 30 s (control)	26.33[a]	0.658	0.21	1.83	40
RNA 37°C 30 s (treatment)	83.84[b]	2.09	0.50	1.87	40
RNA 40°C 7 sec (control)	18.25	0.456	0.076	1.80	40
RNA 40°C 7 s (treatment)	54.38	1.343	0.29	1.83	40
RNA 60°C 6 s (control)	13.40	0.335	0.14	1.77	40
RNA 60°C 6 s (treatment)	32.05	0.801	0.34	1.79	40
RNA 80°C 5 s (control)	14.37	0.359	0.41	1.89	40
RNA 80°C 5 s (treatment)	40.76	1.019	0.71	1.83	40

[b] is significantly higher than [a] with $p<0.05$

TABLE 2. Gene expression data

Thawing temperature/time	Gene	Ct value (mean±SEM)	Relative gene expression (mean±SEM)
RNA 20°C 13 s (control)	HSP70	29.912±1.456	0.036±0.011
	GAPDH	23.745±0.410	
RNA 20°C 13 s (treatment)	HSP70	29.827±1.448	0.054±0.025
	GAPDH	23.920±0.589	
RNA 37°C 30 s (control)	HSP70	29.664±0.568	0.028±0.007[b]
	GAPDH	24.183±0.379	
RNA 37°C 30 s (treatment)	HSP70	26.766±0.269	0.097±0.027[a]
	GAPDH	23.091±0.414	
RNA 40°C 7 sec (control)	HSP70	29.151±0.564	0.081±0.025
	GAPDH	25.04±0.423	
RNA 40°C 7 s (treatment)	HSP70	26.944±0.177	0.041±0.001
	GAPDH	22.341±0.175	
RNA 60°C 6 s (control)	HSP70	29.123±0.436	0.048±0.008 [b]
	GAPDH	24.616±0.178	
RNA 60°C 6 s (treatment)	HSP70	27.348 ±0.112	0.067±0.009[a]
	GAPDH	23.388±0.142	
RNA 80°C 5 s (control)	HSP70	28.836±0.183	0.07±0.023
	GAPDH	24.627±0.429	
RNA 80°C 5 s (treatment)	HSP70	27.619±0.234	0.038±0.005
	GAPDH	22.857±0.219	

[a] is significantly higher than [b] with $p<0.05$



M = 100 bp DNA ladder. Lane 1 = PCR products from sperms sample in control group thawed at 20°C. Lane 2 = PCR products from sperms sample in control group thawed at 37°C. Lane 3 = PCR products from sperms sample in control group thawed at 40°C. Lane 4 = PCR products from sperms sample in control group thawed at 60°C. Lane 5 = PCR products from sperms sample in control group thawed at 80°C. Lane 6 = PCR products from sperms sample in treatment group thawed at 20°C. Lane 7 = PCR products from sperms sample in treatment group thawed at 37°C. Lane 8 = PCR products from sperms sample in treatment group thawed at 40°C. Lane 9 = PCR products from sperms sample in treatment group thawed at 60°C. Lane 10 = PCR products from sperms sample in treatment group thawed at 80°C

FIGURE 2. Analysis of qPCR products from control and treatment groups for various thawing temperatures. Estimated product sizes are 196 base pair

TABLE 3. Mean percentage of uncapacitated and capacitated sperm in control and treatment group

	Uncapacitated sperm percentage (%) mean	Capacitated sperm percentage (%) mean
20°C 13 s (control)	54.50	36.17
20°C 13 s (treatment)	47.83	31.33
37°C 30 s (control)	22.33[a]	61.83[b]
37°C 30 s (treatment)	50.67[b]	28.33[a]
40°C 7 s (control)	23.5[a]	60.33[b]
40°C 7 s (treatment)	41.5[b]	39.33[a]
60°C 6 s (control)	20.59[a]	64.50[b]
60°C 6 s (treatment)	52.33[b]	29.67[a]
80°C 5 s (control)	34.5	52.00
80°C 5 s (treatment)	39.5	40.50

[b] is significantly higher than [a] with $p<0.05$

DISCUSSION

The survival of spermatozoa after freezing-thawing process depends on the plasma membrane as it is the most crucial and regarded as the primary site of cryoinjury (Söderquist et al. 1997). There are a few techniques for sperm preparations before cryopreservation and one of the latest technique is using magnetic separation. Sample form MACS procedure had proven that their membranes are more intact as there were no phosphatidylserine being exposed (Degheidy et al. 2015). 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). Externalisation of plasma membrane Phosphatidylserine (PS) during apoptosis is an oxidative signalling pathway which is identified as one of the earliest and prominent features of apoptosis. In present study, sperms were magnetically labelled with Annexin V microbeads and by-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 good and viable cells were collected for cryopreservation (Dirican et al. 2008). According to Gilbert et al. (2007), ejaculated spermatozoa were

reported to contain 0.0018 pg of RNA per spermatozoon. RNA extraction is a challenging procedure to perform in spermatozoa. This might happen due to the highly differentiated and specialized cells along with the sturdiness of the sperm's membrane itself (Roostaei et al. 2008). Even though sperm is a single and simple cell, its role and composition can suit any challenges during its way to fertilize the oocyte, transporting its paternal genome. One of which is by having an intact sperm cap called acrosome that contains a lot of PUFA to facilitate sperm's fluidity and also the principle piece that aids the movement towards the oocytes. Present study showed only a smear of RNA when visualized on agarose gel after the extraction process. This observation was in agreement with previous reports (Grunewald et al. 2005; Ostermeier et al. 2002). According to Grunewald et al. (2005), mature spermatozoa do not transcribe novel RNA. A smear of RNA obtained during an extraction from human spermatozoa indicated that spermatozoa were free of distinct bands of 28S and 18S ribosomal RNAs. The present result showed that between the control and treatment groups, RNA yield were more abundant in the treatment groups for all thawing conditions. Furthermore, RNA concentration in treatment group was the highest when thawed at 37°C for 30 s with the concentration of 83.84 ng/μL and 1.87 of purity at the wavelength of $A_{260/280}$. This showed that at 37°C, RNA recovery was at its maximum. It is well-accepted that cells are functioning optimally at body temperature which is 37°C. This showed that by combining MACS separation system and thawing temperature at 37°C for 30 s, it yields higher RNA concentration. Normally, multi-cell organisms react to heat or other stresses by inducing the synthesis of unique proteins groups generally referred to as heat-shock proteins or HSPs. Previous studies stated that HSP70 plays an important role as an indicator of thermo tolerance and as a molecular chaperone that functions in aiding and repairing in protein formation (Guo et al. 2010; Piterková et al. 2013). Hence, by quantifying the expression of HSP70 gene, can be directly relating to the semen quality, particularly on its capacitation status. In ANOVA test, we observed that the expression of HSP70 gene were significant at 37°C for 30 s and at 60°C for 6 s with $p<0.05$ in treatment groups. This is probably due to the fact that the RNA concentration in treatment groups were higher as compared to the control group. Moreover, when temperature is higher than 37°C, protein denaturation will start to occur and eventually lead to the degradation of this protein. Contri et al. (2010) and Monterroso et al. (1995) found out that those bull spermatozoa that were exposed to heat shock at 42°C and 43°C have a decreased viability and motility as compared to the lower temperature of 39°C. The ability of HSP to maintain cell survival was detected by the inhibition of apoptosis marker; The Caspase Activation. HSP can impact the 'intrinsic'; the mitochondrial-dependent pathway and the 'extrinsic'; death receptor-mediated pathway of apoptosis. Hence, it is suggested that thawing cryopreserved sperms at 37°C for 30 s, this procedure can facilitate the HSPs to function properly as a chaperon and non-apoptotic role. It is now recognized that the surviving population of spermatozoa is compromised as a result of capacitation-like changes (Bailey et al. 2000; Yeste 2016). In the present study, capacitation status of MACS treated group and control group were assessed using CTC assay. The results indicated that treatment groups produced more uncapacitated sperm as compared to the control group after subjected to different thawing regimes. During the capacitation and acrosomal reaction, PS did not expose on the surface of viable sperm cells. Kurz et al. (2005) mentioned that PS is localized in the cytoplasmic leaflet of the plasma membrane as well as on the outer acrosomal membrane. This clarified and proven current result that during the capacitation process and acrosomal reaction, more uncapacitated cells were revealed after MACS separation. The results showed that thawing at 37°C, 40°C and 60°C yield significant results in treatment groups. Thawing at either one of these temperatures yields spermatozoa that is yet to be capacitated because the process of capacitation must occur at exactly specified time inside the female oviduct tract in order for fertilization to occur. Any event preceding fertilization that occurs at the wrong time and the wrong place will results in poor fertilization rates (Simon et al. 2011). Membrane destabilization causes the sperm to exhibit cryo-capacitation with B pattern that favour calcium influx to the cell during cryopreservation (Maxwell & Johnson 1997). Moreover, the capacitation process is believed to have a relationship with apoptosis. Reactive oxygen species (ROS) are essential in regulating sperm function, but it 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. However, excessive production of ROS can lead to a state of oxidative stress that affects sperm function.

Conclusion

In conclusion, the quality of bull's spermatozoa can also be accessed through the quantification of the HSP70 gene expression rather than the motility alone. Expression of this gene coupled with the capacitation status concluded that the MACS is an ideal sperm preparation technique prior to cryopreservation. By choosing the suitable thawing temperature which is at 37°C for 30 s, the spermatozoa population produced is at its highest HSP70 gene expression and creating less capacitated sperms. However, the fertility potential of this MACS sperm population has yet to be determined. This could be achieved through further research in the future.

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