International Journal of Engineering & Technology, 7 (4.14) (2018) 127-132

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International Journal of Engineering & Technology

Website: www.sciencepubco.com/index.php/IJET

Research paper



Analysis of Bovine Spermatozoa *CSP*-Gene Expression using qPCR and Relative Quantification Method as Biodiagnostic Tool for Fertilizing Capacity

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Abstract

Gene expression studies enable for real-time relative quantification of expressed genes. However, the incorporation of a primary discerning tool before amplification for specific sequences of genes of interest is yet to be implemented. The amplification of gene sequences from discriminate sample groups further enhances the results following qPCR and provides an absolute exclusive measurement for the defined sample. Cryopreserved spermatozoa have characteristics of compromised sperm quality as the cells are exposed to a rapid temperature downshift not normally encountered by such cells *in vivo*. Thus, the use of a separation system before cryopreservation produces a refined outcome in gene expression studies. The proliferation of Cold Shock Protein (*CSP*) can relatively be quantified by conducting qPCR following sperm selection and cryopreservation. *CSP*s are indicators of cells adapting to the decrease in temperature and indirectly acts as an intracellular protective mechanism for the cell. The current study compares the effects of sperm selection system in isolating a homogeneous population of spermatozoa for cryopreservation followed by quantification of *CSP* gene. Method of molecular assessment involved quantifying the amplified sequences via qPCR and gene expression following sperm isolation was significant in increasing spermatozoa viability by 26.2% ($P< 0.05$). The relative fold expression of *CSP* gene for treatment group increased from 1.00 fold to 1.38 fold. The Cq values for treatment group had recorded an earlier point of amplification (Cq= 24.87) as compared to control Cq= 25.83. Our findings suggest that the use of a sperm isolation system before cryopreservation would increase the probability of obtaining higher amplifications of *CSP* genes that would confer protection against extremely low temperatures during cryopreservation. This would increase the likelihood of *in vitro* fertilization using cryopreserved spermatozoa by implementing qPCR as a potential biomolecular diagnostic tool to ascertain the fertilizing potential of the spermatozoa.

Keywords: *Biodiagnostic tool; cold schock protein; CSP gene; fertilizing capacity; quantitative real-time PCR (qPCR); spermatozoa*

1. Introduction

The integrity of viable cells is continuously challenged by physical stress and may impart itself of oxygen deprivation, exposure to either high or low temperatures which would result in the expression of stress proteins. As an example, an elevation in temperature produces heat shock protein. Whereas production of cold shock protein occurs when stress proteins are subjected to a sudden drop in temperature as for the case of cryopreservation [1]. Past studies have shown that a positive relationship between the decrease in temperature (exposure to low temperature) and expressed cold shock protein [1]. Cold-shock proteins (*CSP*s) are small (7 kDa) proteins involved in mRNA folding, protein synthesis and/or freeze protection. Yamanaka [2] had revealed that *CSP*s are synthesized in the presence of a downstream box (DB Box) sequence within the mRNA. The location of the DB Box is after the initiation codon in the coding sequence of mRNAs. This scenario is true for all *CSP* categorized in Class I. The sequence of the DB Box is complementary to the anti-DB sequence found in 16s rRNAs. It was asserted to be an independent translational signal enhanced via the formation of the translational initiation complex. The binding of *CSP* to ssRNA inhibits any secondary structures from forming in the RNA as its major role is to sustain the unfolding of RNA at low temperatures. The biological function of this protein has an effect on both the translation of mRNA in the cell and transcription regulation at low temperatures observed post-cryopreservation procedures [3]. Therefore, *CSP* can be regarded as an indicator that the cell is adapting to the increase in temperature and the expression of *CSP* indirectly acts as an intracellular protective mechanism for the cell. To improve the quality of sperm used in the analysis, the spermatozoa are selectively separated from the neat semen by using ISolate® sperm separation medium. The isolation process facilitates *in vitro* fertilization by generating a discrete fraction differentiating the viable sperm cells from the unfavourable cellular debris containing free radical generators which may give effect to the quality and viability of the sperm and propagates clean fractions with leukocytes removed, reduced ROS levels and yields are of optimal quality [4]. The importance of conducting this study is to consider the effects of

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sperm separation on the expression of *CSP* and quantifying the expression via qPCR.

2. Method

2.1. Maintenance of animal and sample collection

Mature Charolais cross Kedah-Kelantan breed (CHX) bulls (36-48 months old) were housed at the Institut Bioteknologi Veterinar Kebangsaan, Malaysia. Sample collection is as previously described in [5] was conducted during March to April upon approval from the institutions animal ethical committee. The ejaculates were separated into 2 groups: (i) control group (cryopreserved sperm without sperm isolation system) and (ii) treatment group with the use of sperm isolation system preceding cryopreservation.

2.2. Semen preparation for Sperm Isolation System

To propagate clean fractions of spermatozoa void of free radical generators, the neat semen was exposed to double density gradient centrifugation (ISolate®, Irvine Scientific, USA). The protocol was in accordance to the manufacturer's instructions. Briefly, equal volumes of Lower Layer (90% density), Upper Layer (50% density) and semen sample were sequentially transferred with a pipette into a microcentrifuge tube. The tubes were centrifuged at 300 RCF for 20 minutes. Following centrifugation, the supernatant was removed, and the pellet suspended in Biox-cell® (IMV, L'Aigle, France) preceding cryopreservation. This homogeneous population of sperm was denoted as the treatment group.

2.3. Sperm Isolation System Cryopreservation and thawing protocol for sperm

Sperm from control and treatment group were cryopreserved in Bioxcell® extender (IMV, France). The initial concentration of the mixture was determined using SpermaCueTM (MiniTube, Germany) and adjusted to 25 X 10^6 cells/mL in 0.25mL cryopreservation straws. Cryopreservation was conducted based on the protocol as described by [6]. Thawing protocols for both groups of sperm were conducted in a 37°C water bath for 30secs.

2.4. Extraction of sperm RNA and cDNA synthesis

Extracted sperm RNA from pooled 20 straws/ group (n=20) of cryopreserved sperm were centrifuged at 200-300 RCF for 15 minutes to form a pellet. The resulting supernatant was discarded and the pellet used as the starting material for RNA extraction using Invitrap® Spin Tissue RNA Mini Kit with DNase digestion. RNA integrity was spectrophotometrically determined at λ 260 nm, 280 nm and 230 nm, using a Nano-Drop spectrophotometer (Thermo-Fischer Scientific). Validation of RNA integrity was conducted by examining the bands corresponding to the RNA subunits via electrophoresis on 0.5% (w/v) agarose gel (5V/cm, 1.5hr). RNA was diluted to a working concentration of 5ng/µL before cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad Laboratories Inc., USA). The reaction vial was prepared by combining 4 µL of 5X iScript buffer, 1 µL iScript reverse transcriptase, 10 µL RNA template and nuclease-free water were added to produce a reaction volume of 20 µL. The cDNA synthesis cycle protocol was programmed as 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 at -20°C until further use.

2.5. Relative quantification of *CSP* gene using qPCR

Primers used in this assay were synthesised using Primer 3 software and BLAST tool at National Centre for Biotechnology Information (NCBI) homepage. Primer for the gene of interest was designed according to *Bos taurus* cold shock domain containing E1, RNA-binding (CSDE1) *CSP* mRNA (Reference Sequence: NM_001098025.1): (5'-3') GACCGGCGGACTGGGAAACC; (3'-5') GCGTGCAAACTGAGGAGATGGGG with an expected product size of 182bp. The primer of reference gene was designed according to GAPDH mRNA (Reference Sequence: XR_027767): (5'-3') CTGAGGACCAGGTTGTCTCC; (3'-5') CCAC-CACCCTGTTGCTGTAG with an expected product size of 197bp. Primer design was also based on the whole genome assembly of domestic cow, *Bos taurus* [7]. qPCR reaction mixture consisted of 1x iQ SYBR Green supermix (Bio-Rad Laboratories Inc., USA), 50ng cDNA, 0.25µM each of forward and reverse primers and RNAse-free water in a final reaction volume of 25µl. The qPCR cycling protocol was programmed as follows: 1 cycle of enzyme activation at 95°C for 30secs, 40 cycles of denaturation at 95°C for 5secs, 40 cycles of annealing/extension at 62°C for 10secs and 1 cycle of melting curve from 55°C - 95°C (in 0.5°C increment) for 10 secs/step. The sample was held at 4°C during cooling. Total reaction time is approximately 2 h and 30 min. The qPCR values were determined from the geometric mean of 2-ΔΔCt of the reference gene: *Bos taurus* glyceraldehyde phosphate dehydrogenase (GAPDH) against *CSP* genes [8]. The relative quantification of gene expression was conducted using the gene expression study module of iCycler iQ Multicolour Real-Time PCR Detection System.

2.6. Determination of DNA Fragmentation with Sperm-Halomax®

The DNA fragmentation assay was conducted in accordance with the manufacturer's recommendations included in the kit. The assay required cell membrane lysis in order to assess the DNA integrity. Thawed sperm at a concentration of 5-10 x 10^6 spermatozoa/ ml was combined with melted agar (equilibrated at 37°C in a water bath) prior to placing on a thermally equilibrated slide and covered with a coverslip. This was left to solidify at 4°C for 5 minutes. Once solidified, the coverslip was removed and the slide was immersed in lysis solution (5min), distilled water (5min), 70% ethanol (2min), 90% ethanol (2min) and 100% ethanol (2min). Slides were stained with 10µl of DNA specific fluorochrome, 50µg/ml ethidium bromide (absorption and emission spectra: 518nm and 605nm respectively) and observed immediately under fluorescent microscopy at 40 x magnification. A total of 300 cells per data entry were observed and enumerated for a percentage of DNA fragmentation.

2.7. Statistical Analysis

Statistical analysis was performed using the SPSS software, version 22.0. The differences in mean of control and treatment groups for sperm *CSP* gene expression and DNA integrity were determined using the Mann-Whitney U Test and considered as significant at $p<0.05$. The relative quantification of gene expression was conducted using the gene expression study on iCycler iQ Multicolour Real-Time PCR Detection System (Bio-Rad Laboratories Inc., USA).

3. Results

3.1. Bovine Sperm RNA

The average concentration of CHX sperm RNA from the control group (cryopreserved sperm without sperm isolation system) is 16.71ng/µl and treatment group (use of sperm isolation system preceding cryopreservation) is 22.82 ng/µl. RNA purity based on A260/280 ratio is 1.88 for control and 1.83 for the treatment group. This shows that the extracted RNA is devoid of contamination and is within the standard. Resolution of extracted RNA show streaking (Fig. 1) and is common for sperm RNA. Sperm only delivers a limited amount of RNA which is estimated to be within

5-10fg per porcine sperm cell [9]; 10-20fg per human sperm cell [10] and only 150pg per bovine sperm cell [11].

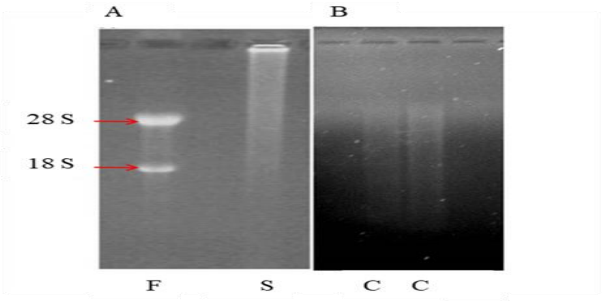

Fig. 1: Resolution of Purified RNA from Human and Bovine Spermatozoa on 0.5% Agarose gel. A: Resolution of purified human RNA; S= mature spermatozoa RNA and F= dermal fibroblast RNA (adapted from [12]; B: Resolution of purified bovine RNA. C= RNA from CHX bulls.

3.2. Sperm DNA integrity

Prior to amplification of the *CSP* gene and quantification of gene expression, the integrity of the sperm DNA was analysed. Fig. 2 (A) is an illustration of spermatozoa with fragmented DNA. The size of the halo exceeds its cellular membrane boundary thus, causing the DNA material to be scattered away from the nucleus of the cell. This reaction ensues as the cellular membrane of the spermatozoa had been lysed and the fragmented DNA is released. The fluorophore used for staining functions in binding to DNA. Therefore, the sperm with fragmented DNA produces a larger halo pattern as the DNA has drifted away from the main sperm body. In contrast, spermatozoa with intact DNA do not produce the distinct halo pattern because its DNA is compact and integrity is maintained. It is evident from Fig. 2 (A), that the occurrence of spermatozoa with fragmented DNA is more common in the control group (cryo-preserved sperm without sperm isolation system) as compared to the treatment (use of sperm isolation system preceding cryopreservation) (Fig. 2 (B)). As shown in Fig. 2 (B), despite sufficient lysis of the cellular membrane the DNA maintained at the core of the cell suggesting that DNA content is intact. The results of the Mann-Whitney U test showed a z score of -4.048, $p < 0.01$ and the control group of spermatozoa had an average rank of 24.07, while treatment group had an average rank of 9.75. This is evidence that treatment group has conserved DNA integrity.

Table 1: Analysis of DNA Fragmentation in Control and Treatment sub-population of Spermatozoa using SPERM-Halomax®

Spermatozoa sub-population	Percentage of DNA Fragmentation	Mean Rank	p value
Control	1.52 ± 0.22	24.07	<0.01
Treatment	0.17 ± 0.58	9.75	<0.01

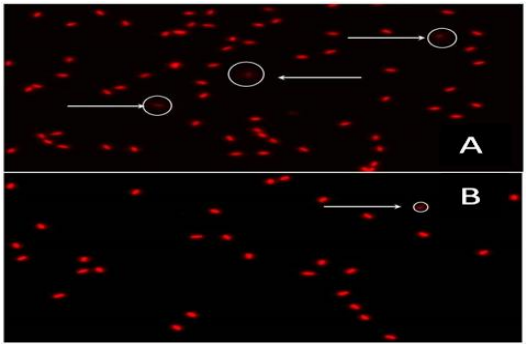

Fig. 2: The spermatozoa exhibiting fragmented DNA is labelled with an arrow and encircled. The images were taken at 40x magnification. A: Observation for DNA integrity showed more occurrences of fragmented DNA in control group (cryopreserved sperm without sperm isolation system). B: DNA was intact in the treatment group (use of sperm isolation system preceding cryopreservation) and showed less occurrence of halo.

3.3.*CSP* Gene expression using qPCR

The standard curve (Fig. 3) generated as a reference for primer's efficiency rate recorded an efficiency rate of 115% and R^2= 0.996 which fulfils the requirements as outlined in the MIQE Guidelines for qPCR [13]. The factor dilution of the primer was a 4-fold dilution factor.

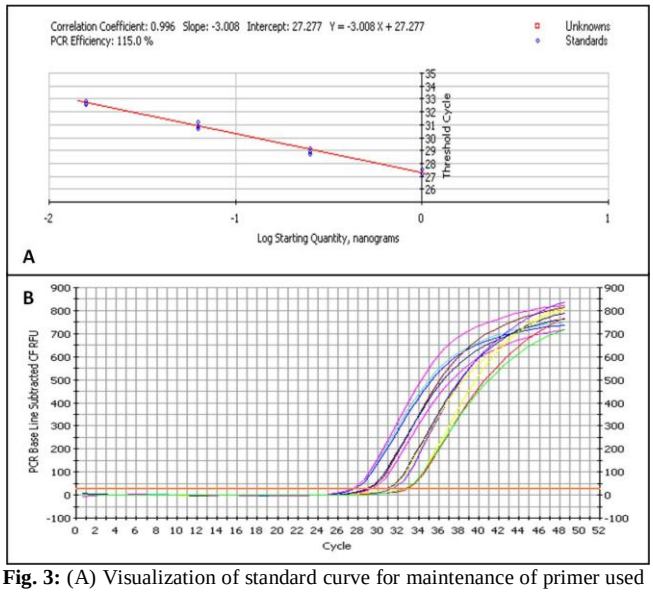

Fig. 3: (A) Visualization of standard curve for maintenance of primer used in qPCR analysis in the study. (B) Amplification curve generated from qPCR analysis for standard curve of primer.

Table 2 is the qPCR analysis generated for *CSP* gene and GAPDH reference gene in both (i) control group (cryopreserved sperm with-out sperm isolation system) and (ii) treatment group with the use of sperm isolation system preceding cryopreservation. qPCR observations show an earlier point of amplification for *CSP* gene in the treatment group (Cq: 24.87) as compared to control group (Cq: 25.83) which suggests that there is a greater expression of the gene following a sperm isolation process before cryopreservation. It is also evident that this increase in expression level is responsible for the conserved DNA integrity within sperm in the treatment group.

Table 2: Cycle number (Cq) amplification data, melting temperature (T_m) from melting curve and gene expression data for *CSP* gene and GAPDH reference gene.

Group	Gene expression (Cq value)	T_m (°C)	Relative fold expression
GAPDH gene (reference gene for control group)	20	83	1.00
GAPDH gene (reference gene for treatment group)	20	83	1.10
CSP gene (cryopreserved sperm control group)	25.83	83	1.00
CSP gene (isolated and cryo-preserved sperm treatment group)	24.87	85.5	1.38

It is observed that the treatment group recorded an accelerated increase in gene expression levels as compared to the control group for both GAPDH and *CSP* genes. Furthermore, melting temperature (T_m) of reference gene (GAPDH) amplicon was re-

corded at point 83°C. On the other hand, the T_m of *CSP* gene in treatment group amplicon occurred at the inflection point of 85.5°C. The higher T_m suggests a G-C rich DNA sequence in the DNA of the sperm from treatment group thus is thermally stable and may possess base variance [14]. Relative gene expression values have also shown an increase in expression for *CSP* gene from a value of 1.00 fold for reference gene, GAPDH to 1.38 fold for *CSP* gene in treatment group. Analysis of intrasample variations denoted as mean Cq ± SD, CV% showed that Cq of *CSP* gene is 26.09 ± 1.86, CV= 7.13% and Cq of reference gene is 22.89 ± 1.08, CV= 4.72%. Calculation for the expression level of *CSP* gene resulted in an increase by 0.25 fold in comparison to the level of expression of reference gene GAPDH.

4. Discussion

Decline in male fertility has been linked to a plethora of contributing factors. *In vitro* analysis on RNA from sperm destined for assisted insemination following cryopreservation is key in understanding the effects of the expression of *CSP* gene during exposure to extremely low temperatures. This study has reported that an increase in the expression of *CSP* gene has maintained the integrity of the sperm DNA and consequently its physiology and fertilizing capacity (inferred from sperm motility, velocity and progression values previously reported by our lab [5]. *CSP* is a natural cellular mechanism that initiates production of specific stress proteins acting as chaperone proteins which functions in rectifying the potential damage caused by protein misfolding due to temperature fluctuations. *CSP* functions as an intracellular thermometer regulating its own expression responding to temperature and leads to modification in levels of gene expression [1; 15]. Past studies have shown a positive relationship between the decrease in temperature (exposure to low temperature) and cold shock protein expression [16]. In the event of extreme decline in temperature, the transcriptional anti-terminator mechanism leads to an increase in transcription of protein. The increase in protein concentration (including *CSP*) confers protection to the cell because it reduces the possibility of intercellular ice nucleation and crystallization, which would otherwise damage the cell [17]. It has been noted that with a reduction in temperature, good-natured healthy sperm expresses *CSP* that functions in reducing RNA unfolding by rapidly modulating the pattern of protein synthesis in response to fluctuations in the environment. This chaperoning effect, enhances protein translation during temperature downshifts [18]. One of the major protein domains linked to male infertility following cold shock is the CSDE1 domain for *Bos taurus* (Gene ID: 513993). It encompasses 4 conserved domains of which 3 domains are relevant to the viability of the spermatozoa following extreme temperature downshift: *CSP* (cold Shock Protein) domain, CSD (Cold-shock Binding) domain and S1-like ribosomal protein domain. Ribosomal protein S1-like is a RNA-binding domain and is found in a wide variety of RNA-associated proteins. The protein was originally identified in S1 ribosomal protein and also contains the Cold Shock Domain (CSD), a homolog of the S1 domain. Moreover, E1 protein has been extracted from the seminal plasma of males [19] and is a male fertility marker linked to sperm motility, however, has an insignificant effect in sperm concentration and morphology [20]. The domain is located on chromosome 3 with an exon count of 21 and is highly conserved in humans among other available species. Genes important for spermatogenesis have been found on chromosome 3 in mammals and mice [21]. The RNA binding motif protein (RBM) is exclusively expressed in germ cells [23] and a partial deletion in the gene has been linked to azoospermia or severe oligospermia in males [22]. Therefore, translocations in chromosome 3 would affect the fertilizing functionality of the sperm as it is thought to serve as a splicing regulator during spermatogenesis thus directly affects the outcome of the process. Another gene implicated in male infertility is the DAZ gene that is implicated in azoospermia in humans [24]. However, analogues of this gene have been reported in mammals and mice, also on chromosome 3 [21]. Despite the known effects of fluctuations of gene expression during extremely low temperatures, seasonal variations in expressions of genes on male fertility in high temperatures such as the report by [25] on expressions of Heat shock proteins (HSP) in Indian bulls has also shown the same outcome of reduced male fertility. Furthermore, translocation of genes could also have a compounding effect. [26] reported that balanced reciprocal translocation of genes may be the cause of infertility in normozoospermic patients.

The higher T_m recorded in this study suggests a G-C rich DNA sequence which could lead to the base variance that could also be implied in translocation of genes. Results of the DNA fragmentation analysis revealed that 1.52% of the control group sub-population exhibited fragmentation. The treatment group scored nine times less the amount of the control group with only 0.17% DNA fragmentation present (Table 1). The integrity of the mammalian spermatozoa DNA material is of prime importance for paternal genetic contribution to future progeny. It is therefore imperative that DNA integrity is conserved by reducing the impact of handling, processing, cryopreservation and thawing on the sample. Correlations between the presence of nuclear DNA alterations in mature spermatozoa and poor sperm parameters or impaired reproductive efficiency is reported in animals and humans [27]. DNA fragmentation analysis showed that spermatozoa from the treatment group sustained minimal damage to their DNA following separation and cryopreservation, thus also recorded an increase in expression of *CSP* gene which confers protection against damages to sperm membrane, function and physiology due to cold shock to abstain from adverse effects. Cold shock induces an increase in DNA superhelicity and compaction occurrence as well as elevated concentrations of trehalose [28]. For a cell to survive cryopreservation and maintain structural and biochemical functionality as well as fertilizing capacity, it must indicate that the spermatozoa possess self-repair mechanisms compressed within the DNA. These self-repair mechanisms can overcome the deleterious effects of the cryopreservation process [29]. During the cryopreservation of the spermatozoa, the sub-population which did not undergo separation (control group) exhibited higher levels of DNA fragmentation as compared to the sub-population separated through ISolate® system (treatment group). The difference in observed values for fragmented DNA was nine times higher in the control group. This observation could be explained by the fact that the treatment group possessed higher compaction in DNA as the spermatozoa were pelleted at the base of the discontinuous ISolate® gradient at 90% density. Evidently, the density of the spermatozoa would have been greater than the 90% density of the lower layer suggesting heavier spermatozoa bearing amassed DNA compaction [30].

With the induced effect of cryopreserving temperatures, the expression of *CSP* gene has increased as compared to the dormant expression of inert endogenous reference genes, GAPDH. The treatment group in all cases of post-run analysis showed an earlier point of inflection for Cq values as compared to control group. From the melting curve, it can be inferred that there are no contaminating DNA or primer dimers present in the reaction. This is justified by the fact that there is only a single peak in the plot denoting the desired amplicon. Nonetheless, the high efficiency rate of the standard curve plot is characteristic of high variability of RNA at low concentrations and could also be due to PCR inhibition by reverse transcriptase [31]. However, the correlation of relationship coefficient (R^2) relates the relationship between RFU and Cq values and depict the degree of predictability to be very strong. The y-intercept 27.277 of the equation corresponds to the Cq value for a single copy of *CSP* molecule which is relatively accurate and proximate the actual recorded value of 26.09 ± 1.86, CV= 7.13%. The recorded accelerated Cq values are evident of the relationship between Cq value and the amount of nucleic acid in the sequence of interest. Cq levels are inversely proportional to the amount of target nucleic acid in the sample. Therefore, the

lower the Cq level the greater the amount of target nucleic acid within the sample. This further validates the results observed in this study where the treatment group recorded a lower Cq value as compared to the control group. The treatment group that had a sperm separation analysis incorporated into the method showed that with the increase of intact DNA in the sperm population, the higher the expression of *CSP* gene was observed from the lower Cq value obtained from qPCR analysis.

Data of gene expression study is a reflection of spermatozoa physiology. A significant example would be capacitation of spermatozoa which is highly dependent on protein control. By having populations of spermatozoa with intact DNA (minimal or devoid of DNA fragmentation), capacitation can take place naturally within the female reproductive tract thus conferring enhanced fertilization potential in order to penetrate the oocyte. This process can only be achieved through the functional maturation of spermatozoa characterised by the transformation of the plasma membrane via the removal of surface glycoprotein layer and exposing important receptors for the cascade of reactions. This process leads to changes in the acrosomal cap responsible for acrosome reaction leading to hyperactivation [32-33]. This enables the spermatozoa to penetrate the zona pellucida layer of the oocyte in order for fertilization to occur. Therefore, it is imperative that premature hyperactivation is avoided *in vitro* to ensure success in fertility. As earlier discussed, the activated spermatozoa produced from the treatment group sub-population provides a platform for further manipulation prior to insemination into the female reproductive tract. [34] reported that in contrast to capacitation, hyperactivation is independent of protein content. Therefore, by studying the expression of *CSP* following exposure of spermatozoa to extremely low temperatures during cryopreservation, a hypothesis can be indirectly postulated regarding the capacitation state of the cell. It is important to note that *CSP* synthesis is independent of conventional protein synthesis pathway and is not affected by inhibition with antibiotics but can only be blocked by amino acid starvation [35]. Cold shock confers resistance to stress caused by antibiotics, thus enabling the cell to continue the expression of *CSP* at conditions otherwise would block protein synthesis. Furthermore, it was observed by [36] that differing *CSP* proteins possess different ssDNA specificity and mechanism of interactions. It was concluded that these proteins may have different individual functions in their efforts to maintain the survivability of the cell under extreme thermal conditions. This results in better adaptability by the spermatozoa to sustain viability and functionality within its own terms and in the long run, preserve its ability in the expression of *CSP* which also aids in protecting the spermatozoa. As a conclusion, the use of *CSP* gene expression as a biodiagnostic tool for initial determination of the fertilizing capacity of cryopreserved sperm is highly recommended.

5. Conclusion

The initial hypothesis for the study of *CSP* expression outlined that the expression of *CSP* will be upregulated following selection of spermatozoa with ISolate® prior to cryopreservation in efforts of ensuring that only the best of the population is used for the study. Results have shown that there is a slight increase in fold expression of *CSP* within the treatment sub-population. Although the increase in fold expression is not substantial (and at present is insignificant), it, however, provides a promising outlook on how the degree to which the gene is upregulated can further be improved with differing manipulative variables in future studies.

Data had also revealed that the physiological function of the spermatozoa is intact. Thus proves that the intact spermatozoa possess self-repair mechanisms able to surmount the deleterious effects of the temperature downshift. The self-repair mechanism that is located within intact DNA sustained by the selected spermatozoa is corroborated by the results of DNA fragmentation, which had shown a significant reduction of nine-fold difference in percentage of fragmented DNA for the treatment group. It is important to note that *CSP* expression can be used as a diagnostic tool for fertilizing capacity in assessing the translational regulation of protein synthesis in conferring adaptive protection towards external damages in the form of extremely low temperatures. The significance in governing these processes is vital in eukaryotes as it controls the physiological processes of cell development, differentiation and proliferation. Additionally, translational regulation governs in repressing the effects of external damages to the cell by conferring protection and also in metabolic pathway regulation. Therefore, by understanding the underlying mechanism which *CSP* expression has control over in the cell, it can be rendered beneficial to the functional physiology and survivability of the cell following cryopreservation such as in the instance of Assisted Reproductive Technology.

Acknowledgements

Authors would like to thank Prof. Dr. Noorzan Mohd Noor (Professor of linguistics) for proof reading the manuscript and Zawawi Ismail and Nurul Afiza of Institute Bioteknologi Veterinar Kebangsaan (IBVK), Malaysia for technical support during semen collection. Also to Assoc. Professor Dr. Gabriele Ruth Anisah Froemming and Norita Salim of Institute for Medical Molecular Biotechnology (IMMB) of Universiti Teknologi MARA (UiTM), Malaysia for their assistance in providing access and technical support for gene expression study. This research was supported by the grant from the Excellence Fund of UiTM and Research University Fund (UKM) under the purview of Ministry of Education Malaysia. Acknowledgement also goes to Research Management Centre, UiTM and the Faculty of Applied Sciences, UiTM Shah Alam for their support.

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