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Original Article

Albumin Improved Spermatozoa Quality and DNA Integrity For Freezing-Free Preservation

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ABSTRACT

Freezing-free preservation provides a simple yet efficient alternative to semen preservation. Our study objectives were to determine optimum preservation temperature and bovine serum albumin (BSA) concentration for spermatozoa storage. It was conducted in two phases. First phase involved storage temperature optimization through viability assessment. Temperatures used for this phase of the study were 4, 25 and 37 0C. The second phase involved the use of identified optimized temperature in determining optimum BSA concentration. This is achieved through the effect of BSA on viability and DNA integrity. Concentrations that were used to achieve this objective were 0, 1, 4, 8, 12, and 16 mg/ml BSA. Our results showed that optimum temperature was at 40C. By using ANOVA analysis, our optimum BSA concentration for improving viability and DNA integrity were at 8 mg/ml BSA. Multiple regressions test showed that viability percentage can actually be predicted by storage temperature and time using this equation model: Viability = (-0.839)(Time) + (-0.269)(Temperature) + 44.806. Our study had also indicated that for bovine spermatozoa stored at 40C, prediction on viability was influence solely by storage time regardless of BSA concentration, with Viability = (-0.612)(Time) + 33.752. Bovine spermatozoa DNA integrity, however, can be predicted by both BSA concentration and storage time, with Spermatozoa DNA Damage = (0.714)(Time) + (-0.161)[BSA] + 2.411. In conclusion, optimum freezing-free preservation temperature is at 40C with 8 mg/ml BSA supplementation. (231 words)

1. Introduction

In selected primates and non primates, low birth count becomes an alarming health problem. List of endangered animals increases each year because of illegal poaching, indiscriminate killing of animals, infertility and decline of species gene pool. Various advanced reproductive techniques (ART) used aggressively to overcome this problem. These techniques include intracytoplasmic sperm injection (ICSI), artificial insemination (AI), in vitro fertilization (IVF), and interspecies embryo transfer.

The development of these techniques allowed the repopulation of endangered cattle species namely the Bali (*Bos javanicus*) cattle indirectly . Further development of reproduction techniques also allowed the development of new cattle subspecies with specific characteristics. The selective crossbreeding also allowed the growth of the specialized feed lotting industry indirectly. This is evident with the advancement of bovine embryo transfer that had resulted into a large international business that had expanded dramatically in just three decades. Currently it has been reported that more than 500,000 embryos are produced yearly from superovulated cow worldwide . Despite the advancement in reproductive techniques, innovation in cryopreservation has been at a standstill for the past two decades.

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Conventional method for long term spermatozoa preservation involved freezing of spermatozoa in liquid nitrogen at -196˚C . However, this method poses some limitations especially if cryopreservation is to be carried out at remote farm or across another part of the country. Need of liquid nitrogen, handling and logistic difficulties makes cryopreservation hard to perform at small scale farm especially in developing country, as well as in deep jungle for wildlife animal conservation purposes .

Natural pregnancy is not possible if the spermatozoa have extensive DNA damage. DNA damage in cryopreserved spermatozoa is negatively correlated with its fertilizing potential. Not much is known about the effect of cryopreservation on spermatozoa. However, studies have conclusively shown cryopreservation procedure results in reduction of spermatozoa fertilizing capability. All this arises both from a lower post-thawed viability and sublethal dysfunctions of the surviving spermatozoa population. This is due to cryocapacitation damage following freezing and thawing process .

Freezing-free preservation has been postulated to be able to lessen damage to spermatozoa compared to a freezing protocol. Even though this preservation can results in production of normal offspring after ICSI procedure, duration of preservation using this method has to be improved because it only allows short term storage . This has been supported by that showed unfrozen spermatozoa can fertilize an oocyte after seven or eight days storage based on the media used. Improvement in this field can be done by discovering ideal storage temperature as well as supplementation of compound that can maintain and improve quality of stored spermatozoa. Antioxidant is usually added into preservation media for improvement of spermatozoa quality. Coenzyme Q10 (CoQ10), alpha lipoic acid (ALA) , BSA are some of the well known antioxidant used for improvement of spermatozoa quality.

Our current study is the extension of the previous study, in which cryopreserved Bos javanicus semen were thawed and stored at above freezing point temperature with various BSA concentrations. In our previous report, study had suggested that BSA had indeed improve viability of stored spermatozoa for up to seven days . However, as cryopreserved spermatozoa were used, osmolality effect of BSA was suspected to cause an additional lethal effect on the stored bovine spermatozoa. To avoid this problem, fresh bovine semen samples were used instead of cryopreserved spermatozoa in this study.

2. Material and Methods

2.1. Sample preparation

Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) approved research procedures in this study. Trained personnel involved in all semen sampling done. Fresh semen sample from adult Piedmontese cattle were collected through artificial vagina technique. Six ejaculates were used for each of the evaluation. After collection, all of the semen samples were pooled and determination of spermatozoa concentration was carried out through the use of Spermacue™ with microcuvette (Minitube, USA). Semen samples were diluted in Bio-excell extender to a final concentration of 25 x 10^6 sperm/ml. After dilution, samples were then evaluated for quality.

2.2. Determination of optimum temperature for bovine spermatozoa freezing-free preservation for 31 days

After extension of volume, bovine semen sample were then divided into three main groups: 4, 25 and 37˚C group. Each of the semen samples was stored according to the storage temperature. Viability assessment was used for determination of optimum storage temperature. Viability assessments of fresh sample were carried out and data obtained were treated as baseline data. Day one assessment was carried out after 24 hours incubation. Subsequent viability assessment was carried out every three days until day-31. Optimum storage temperature obtained from this experiment was later used for determination of effects of BSA concentration on spermatozoa viability and DNA integrity.

2.3. Determination of optimum BSA concentration for spermatozoa viability and DNA integrity for 28 days

In this objective, optimum temperature from previous experiment was used for storage of bovine spermatozoa. After extension and pooling of fresh bovine semen, samples were then assessed for viability and DNA integrity. Fresh sample data were treated as baseline data. After assessment, semen sample were then divided into two main groups: treatment group with BSA (1, 4, 8, 12 and 16 mg/ml BSA), and control group without BSA. Both of the groups were incubated for 24 hours before day-1 assessment was carried out. Viability and DNA integrity assessment were carried out every seven days after day-1, for up to 28 days.

2.4. Viability Assessment through Modified Hypoosmotic Swelling Test

Hypo-osmotic swelling test used in this study was a modification of . Briefly, 500 µl of hypo-osmotic solution (0.735 g trisodium citrat dihydrat and 1.351 g D (-) Fructose in 100 ml dH20) was added into 50 µl of fresh semen sample. After addition, incubation was carried out at 37 ºC waterbath for 45 minutes. A volume of 10 µl sample was smeared on a clean glass slide and air dried. Staining of the colorless spermatozoa to observer presence of swollen tail was done through a the use of Diff-Quik® stain (IVD Company, Switzerland). The stained slides were immediately observed under bright microcope with 100x magnification. One hundred spermatozoa were counted for each of the slide to minimize error variation.

2.5. DNA Integrity Assessment Through Neutral Comet Assay

Neutral comet assay used in this experiment was modification from Boe-Hansen et al. method . Briefly, neutral comet assay involved four main steps: slide preparation, lyses of spermatozoa, neutral electrophoresis, and last but not least, staining of the slides.

During slide preparation step, slides were prepared by applying a thin layer of 250 µl NMPA 1 % on single frosted microscope slides using a pipette tip and cover slipped. Slides were cooled in refrigerator at 4˚C for 30 minutes. After cooling, cover slip were remove and second layer of 10 µl LMPA 0.5 % with semen was applied on top of the NMPA layer and cover slipped. Slides were incubated at 4˚C for another 30 minutes.

The slides were then removed from the refrigerator, immersed into a container containing 500 ml of lyses buffer. The lyses buffer contained 2.5 M NaCl, 100 mM EDTA, 10 mM Tris Base, (pH 10.3), 1% (w/v) Triton X-100, 40 mM DTT and 500 mL of 10 mg/mL Proteinase K. The slides were later incubated for one(1) hour at room temperature, followed by 24 hours incubation at 37˚C. After the incubation, the slides were washed three times at 20 min intervals with double distilled water by transferring the slides from one container to another, with intention to remove traces of salt and detergent. The container was covered with aluminum foil to minimize light exposure.

In neutral electrophoresis step, slides were placed uniformly in columns in the electrophoresis tray. The tray was then carefully placed in a horizontal electrophoresis tank containing a mixture of 1 200 mL dH2O and 300 mL neutral electrophoresis buffer (54 g/L Tris base, 27.5 g/L boric acid, 0.5 M EDTA) at pH 8.0. The surfaces of the slides were covered by 0.5 cm of buffer and the lid was placed on the electrophoresis tank to minimize light. Equilibration was allowed for 20 min before running electrophoresis. After the incubation period electrophoresis was conducted for 20 min at 25 V, 0.01 A. When electrophoresis was completed, the slides were removed from tank and continued with staining procedure.

Slides staining were carried out by applying 10 µl of 50 µg/ml ethidium bromide directly onto the LMPA agar layer, and cover slipped. Slides observation was carried out under fluorescent microscope Olympus BX51 with Cell^A image analyser (Olympus, Japan), with EtBr ex/em: 510/590 nm. Comet Assay CASPLab software Project casp-1.2.2 (University of Wroclaw, Poland) was used for DNA tail moment analysis. One hundred cell were analyzed per slide for DNA damage. Extent of DNA damage was based on DNA tail moment.

2.6. Statistical Analysis

One way analysis of variance (One Way ANOVA) was used to determine the effect of BSA on spermatozoa viability and DNA integrity. Pearson correlation test was used to determined relationship between viability and time as well. Results were reported as mean ± SEM, with $p<0.05$ inidicates statistically significant. On the other hand, Pearson correlation test was used to determine the relationship between storage temperature, storage duration and spermatozoa viability. Multiple regression analysis was also used for prediction of experiments outcome. A significant change was indicated as $p<0.05$, and results were presented in mean ± S.E.M.

3. Results

3.1. Determination of optimum temperature for bovine spermatozoa freezing-free preservation after 31 days storage

After 31 days of storage at above freezing point temperature, our results indicated that 4 ˚C is the optimum temperature for bovine spermatozoa storage. Viability percentages of spermatozoa were significantly higher at 4˚C group compared to other groups throughout the assessment period (Figure 1.1). Pearson correlation showed that there is significant relationship between storage temperature and spermatozoa viability throughout 31 days of storage period (Table 2.1). It is a negative relationship, with lower temperature tend to yield higher viability percentage. On the other hand, Pearson correlation test between storage temperature and storage time (Table 2.2) showed a significant negative relationship, in which lower storage temperature allowed longer storage period of bovine spermatozoa.

Multiple regression analysis of storage time (day), temperature (˚C) and spermatozoa viability (%) showed that both time and temperature played a significant role in the prediction of spermatozoa viability percentage, with $F(2, 177)=307.143$, $p<0.001$. The prediction relationship between viability, time and temperature are summarize in an equation of Viability (%) = (-0.839)(Storage time) + (-0.269)(Temperature) + 44.806. We concluded that 4 ˚C is the optimum freezing-free preservation temperature. This temperature was then used in the next phase of study to determine optimum concentration of BSA for spermatozoa viability and DNA integrity.

3.2. Determination of optimum BSA concentration on viability after 28 days storage at 4˚C

Bovine semen was stored at 4˚C for optimum BSA concentration determination. After one day of incubation, group of 4 mg/ml BSA (33.963 ± 2.011 %) gave higher viability percentage significantly compared to control (26.347 ± 1.110 %) and 1 mg/ml BSA (26.933 ± 1.596 %) groups. Group of 8 mg/ml BSA (38.743 ± 0.854) gave the highest viability percentage significantly compared to the other groups. Bovine spermatozoa in the group of 12 mg/ml BSA (25.998 ± 1.442 %) and 16 mg/ml BSA (26.731 ± 0.511 %) gave lower viability percentage significantly compared to 4 mg/ml BSA and 8 mg/ml BSA groups. Figure 1.2 shows bar-chart for the effect of different BSA concentrations (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa viability (%) after one day storage at 4˚C. The significant value between control and test group was at $F(5, 41)=15.468$, $p<0.001$.

Table 2.0 Correlation for relatioship between storage temperature (4, 25, and 37°C) and bovine spermatozoa viability for 31 days

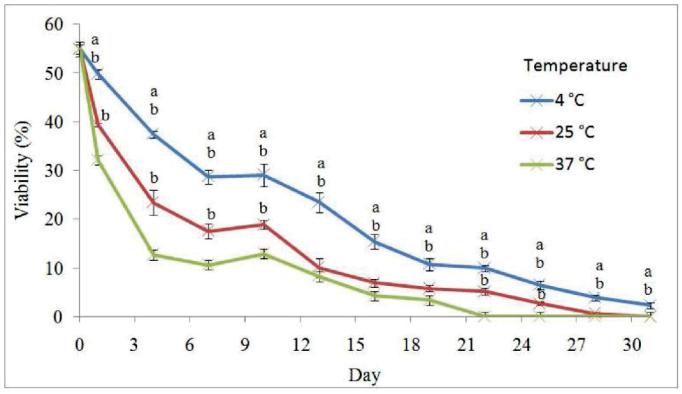
	Day										
	1	4	7	10	13	16	19	22	28	31	
Pearson Correlation (r)	-0.911	-0.949	-0.924	-0.901	-0.815	-0.856	-0.855	-0.966	-0.915	-0.814	-0.0717
Sig.	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003

Table 2.1 Correlation for relatioship between storage temparature (4, 25, and 37°C) and storage time

	Day						
	4	25	25	22	28	31	
Pearson Correlation (r)	-0.952	-0.871	-0.871	-0.786	-0.915	-0.814	-0.0717
Sig.	0.000	0.000	0.000	0.000	0.000	0.000	0.003

At day seven of storage, group of 4 mg/ml BSA (23.161 ± 1.124 %) showed a higher viability percentage significantly compared to control (15.596 ± 0.542 %) and 1 mg/ml BSA (17.423 ± 0.459 %) groups. Group of 8 mg/ml BSA (36.301 ± 1.481 %) gave the highest viability percentage significantly compared to the other groups. While both groups of 12 mg/ml BSA (18.656 ± 1.004 %) and 16 mg/ml BSA (17.723 ± 1.105 %) showed a significant lower viability percentage compared to 4 mg/ml BSA and 8 mg/ml BSA groups. Figure 1.3 shows bar-chart for the effect of different BSA concentrations (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa viability (%) after seven days storage at 4°C. The significant value between control and test group occur at F (5, 41) =55.764, p<0.001.

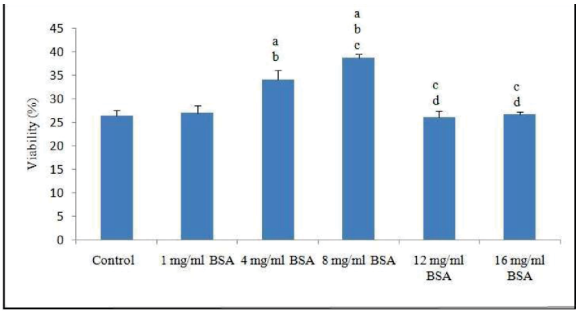
Figure 1.1 Viability progression of bovine spermatozoa stored at above freezing point temperatures for duration of 31 days. Results were presented in mean ± SEM, with p<0.05 indicates statistical significant differences between group.



a indicates higher significantly compared to 25°C group
b indicates higher significantly compared to 37°C group

At day 14 of storage, both group of 1 mg/ml BSA (17.938 ± 0.296 %) and 4 mg/ml BSA (19.689 ± 0.622 %) mg/ml BSA showed higher viability percentage significantly compared to control group (12.327 ± 0.766 %). While group of 8 mg/ml BSA (35.223 ± 0.689 %) showed the highest viability significantly compared to the other groups. Group of 12 mg/ml BSA (17.158 ± 0.398 %) showed a higher viability percentage significantly compared to the control group but lower significantly compared to the group of 8 mg/ml BSA. Group of 16 mg/ml BSA (15.924 ± 0.707 %) showed a higher viability percentage significantly compared to the control group but lower significantly compared to the groups of 4 mg/ml BSA and 8 mg/ml BSA. The significant value between control and test group occur at F(5,41)=219.229, p<0.001. Figure 1.4 shows bar-chart for the effect of different BSA concentration (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa viability (%) after 14 days storage at 4°C.

Figure 1.2. Effect of BSA (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa viability (%) after one day storage at 4 0C. Results were presented in mean ± SEM, with p<0.05 indicates statistical significant differences between group



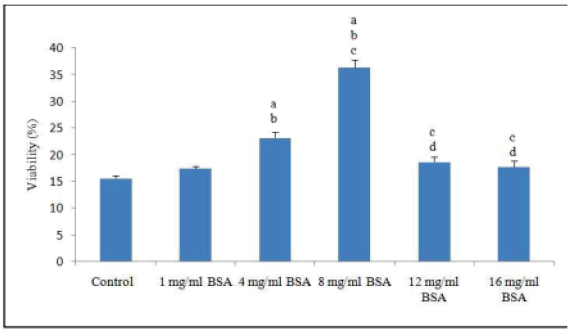
a. indicates significant compared to control group
b. indicates significant compared to 1 mg/ml BSA group
c. indicates significant compared to 4 mg/ml BSA group
d. indicates significant compared to 8 mg/ml BSA group

At day seven of storage, group of 4 mg/ml BSA (23.161 ± 1.124 %) showed a higher viability percentage significantly compared to control (15.596 ± 0.542 %) and 1 mg/ml BSA (17.423 ± 0.459 %) groups. Group of 8 mg/ml BSA (36.301 ± 1.481 %) gave the highest viability percentage significantly compared to the other groups. While both groups of 12 mg/ml BSA (18.656 ± 1.004 %) and 16 mg/ml BSA (17.723 ± 1.105 %) showed a significant lower viability percentage compared to 4 mg/ml BSA and 8 mg/ml BSA groups. Figure 1.3 shows bar-chart for the effect of different BSA concentrations (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa viability (%) after seven days storage at 4 °C. The significant value between control and test group occur at F (5, 41) =55.764, p<0.001.

Figure 1.5 shows bar-chart on the effect of different BSA concentrations (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa viability (%) after 21 days storage at 4°C.

Towards the end of assessment day, day 28, both 1 mg/ml BSA (16.553 ±0.568 %) and 4 mg/ml BSA (18.701 ± 0.394 %) groups showed higher viability percentage compared to control group (11.040 ± 0.654 %). Group of 8 mg/ml (35.125 ± 1.351 %) still gave the highest viability percentage significantly compared to the rest of the group. Group of 12 mg/ml BSA (17.156 ± 0.312 %) showed higher viability percentage compared to control group but lower significantly compared to 8 mg/ml group. On the other hand, group of 16 mg/ml BSA (14.028 ± 1.029 %) gave lower viability percentage significantly to both groups of 4 mg/ml BSA and 8 mg/ml BSA. Significant interaction between control and test group occur at F(4, 51)=108.350, p<0.001. Figure 1.6 shows bar-chart on the effect of different BSA concentrations (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa viability (%) after 28 days storage at 4°C.

Figure 1.3.Effect of BSA (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa viability (%) after seven days storage at 4 °C. Results were presented in mean ± SEM, with p<0.05 indicates statistical significant differences between group.



a. indicates significant compared to control group
b. indicates significant compared to 1 mg/ml BSA group
c. indicates significant compared to 4 mg/ml BSA group
d. indicates significant compared to 8 mg/ml BSA group

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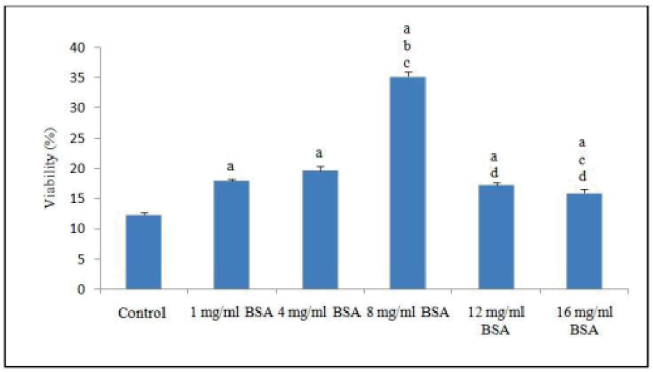
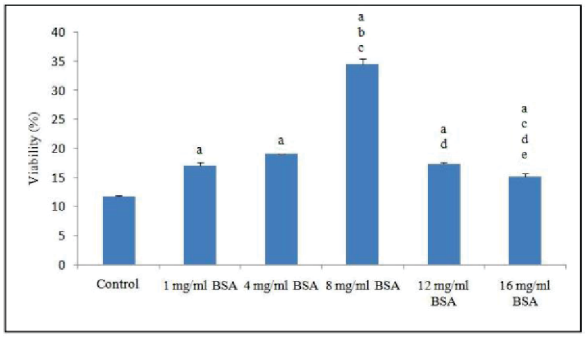
Figure 1.4Effect of BSA (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa viability (%) after 14 days storage at 4 0C. Results were presented in mean ± SEM, with p<0.05 indicates statistical significant differences between group.

a. indicates significant compared to control group
b. indicates significant compared to 1 mg/ml BSA group
c. indicates significant compared to 4 mg/ml BSA group
d. indicates significant compared to 8 mg/ml BSA group



In overall, group of 8 mg/ml BSA gave the best viability percentage compared to other test and control groups. Figure 1.7 shows line graph on spermatozoa viability stored at 4°C for 28 days. Our results show that 8 mg/ml BSA gave significantly higher viability percentage compared to control group throughout the assessment period. Multipe regression test showed storage time plays a significant role in bovine spermatozoa viability percentage prediction, F (2, 279)=84.096 , p<0.001. Viability percentage prediction can be obtained from the formula; Viability = (-0.612)(Time) + 33.752.

Figure 1.5. Effect of BSA (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa viability (%) after 21 days storage at 40C. Results were presented in mean ± SEM, with p<0.05 indicates statistical significant differences between group.

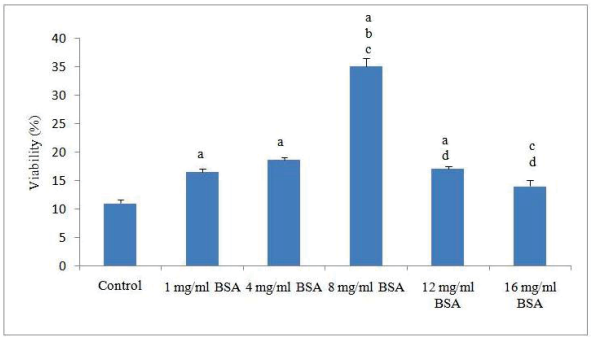


a. indicates significant compared to control group
b. indicates significant compared to 1 mg/ml BSA group
c. indicates significant compared to 4 mg/ml BSA group
d. indicates significant compared to 8 mg/ml BSA group
e. indicates significant compared to 12 mg/ml BSA group

3.3. Effects of different BSA concentrations on spermatozoa DNA integrity after 28 days of storage at 4°C

Tail moment values marked spermatozoa DNA damage. The values inversely related to DNA damage. At day one of storage, only 8 mg/ml BSA group showed lower DNA damage (1.541 ± 0.147 µm) compared to the other groups. Value of F(5, 30)= 16.820, p<0.001 showed significant interaction between control and test group. Figures 1.8 shows the effect of different BSA concentration (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa DNA integrity (µm) after one day at 4 ^{0}C.

Figure 1.6. Effect of BSA (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa viability (%) after 28 days storage at 4 0C. Results were presented in mean ± SEM, with p<0.05 indicates statistical significant differences between group.



a. indicates significant compared to control group
b. indicates significant compared to 1 mg/ml BSA group
c. indicates significant compared to 4 mg/ml BSA group
d. indicates significant compared to 8 mg/ml BSA group

At day seven, all groups gave tail moment value significantly lower than control group (7.814 ± 0.537 %), with F(5, 30)=22.619, p<0.001. All BSA supplemented test groups showed no significant differences between them. Figures 1.9 shows the effect of different BSA concentrations (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa DNA integrity (µm) after seven days at 4 ^{0}C.

At day-14, all of the test groups with BSA supplementation showed tail moment value significantly lower than control group (8.358 ± 0.341 µm), with F(5, 30)=22.619, p<0.001. However, no significant differences were shown between BSA supplemented test group. Figures 2.0 shows the effect of different BSA concentrations (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa DNA integrity (µm) after 14 days storage at 4 0C.

At day 21, all of the test groups except 16 mg/ml BSA group showed lower DNA damage compared to control group (10.647 ± 0.721 µm). Supplementation with 16 mg/ml BSA showed higher DNA damage (9.354 ± 0.814 µm) compared to other test groups, but no significant difference with control group. Significant interaction between groups occur at F(5, 30)=14.520, p<0.001. Figures 2.1 shows the effect of different BSA concentrations (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa DNA integrity (µm) after 21 days storage at 4 0C.

At day 28, both groups of 1 mg/ml BSA (9.789 ±0.988 µm) and 4 mg/ml BSA (7.874 ± 0.894 µm) showed lower DNA damage compared to the control group (15.415 ± 1.289 µm). Group of 8 mg/ml BSA (4.931 ± 0.373 µm) showed lower DNA damage significantly compared to both control and 1 mg/ml BSA group. While both groups of 12 mg/ml BSA (9.615 ± 0.704 µm) and 16 mg/ml BSA (10.820 ± 0.974 µm) showed lower DNA damage significantly compared to control group but higher significantly compared to group of 8 mg/ml BSA. Figures 2.2 shows the effect of different BSA concentrations (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa DNA integrity (µm) after 28 days storage at 40C.

In overall, incubation of 8 mg/ml BSA in the preservation medium gave the best protection to bovine spermatozoa DNA compared to the other concentrations and control groups. Figure 2.3 shows a line graph on progression of bovine spermatozoa DNA damage throughout the 28 days assessment. Our results showed that BSA supplementation at 8 mg/ml BSA significantly reduced DNA damage. Multipe regression test between storage time, BSA concentration, and spermatozoa DNA damage showed that BSA concentration and storage time plays a significant role in prediction of DNA damage, F(2, 213)=122.684, p<0.001. The formula; spermatozoa DNA Damage = (7.14)(Time) + (-0.161)(BSA concentration) + 2.411 obtained the damage related.

Figure 1.7Viability progression of bovine spermatozoa stored at 4 0C for 28 days. Results were presented in mean ± SEM, with a indicates higher significantly compared to control group, p<0.05

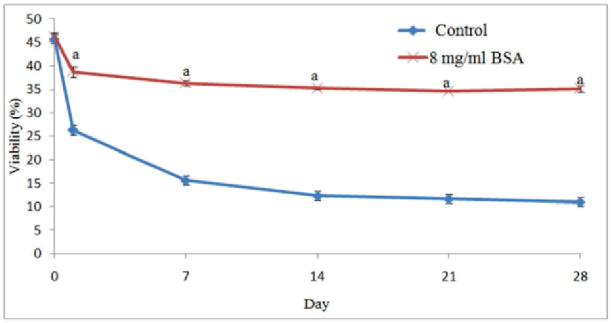


Figure 1.8. Effect of BSA (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa DNA integrity (µm) after one day storage at 4 0C. Results were presented in mean ± SEM, with p<0.05 indicates statistical significant differences between group. a indicates significant compared to control group

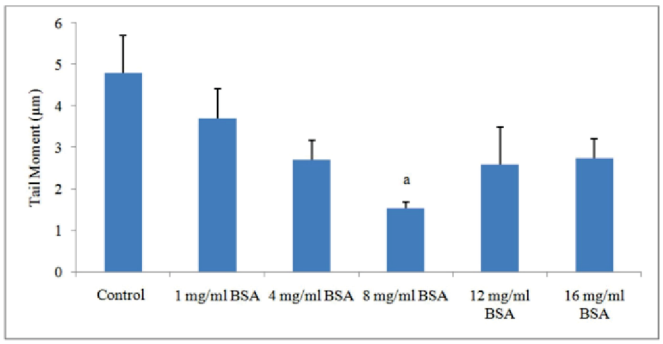
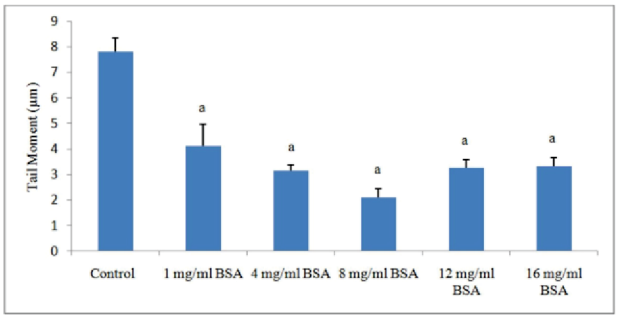
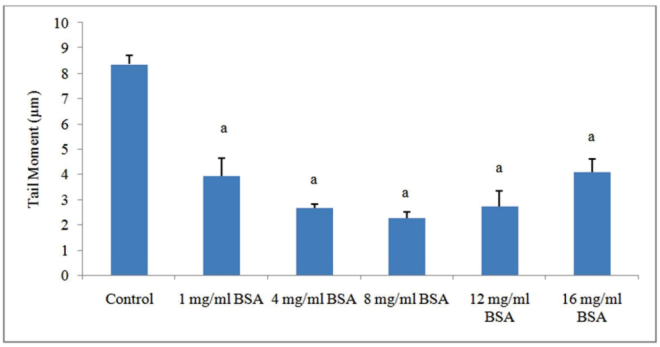


Figure 1.9. Effect of BSA (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa DNA integrity (µm) after seven days storage at 4 0C. Results were presented in mean ± SEM, with p<0.05 indicates statistical significant differences between group.



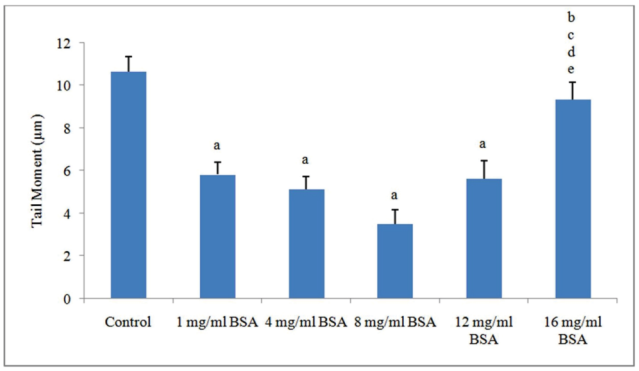
a indicates significant compared to control group

Figure 2.0 Effect of BSA (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa DNA integrity (µm) after 14 days storage at 4 0C. Results were presented in mean ± SEM, with p<0.05 indicates statistical significant differences between group.



a.indicates significant compared to control group

Figure 2.1 Effect of BSA (control, 1, 4, 8, 12 and 16 mg/ml) on bovine spermatozoa DNA integrity (µm) after 21 days storage at 4 0C. Results were presented in mean ± SEM, with p<0.05 indicates statistical significant differences between group.



a.indicates significant compared to control group

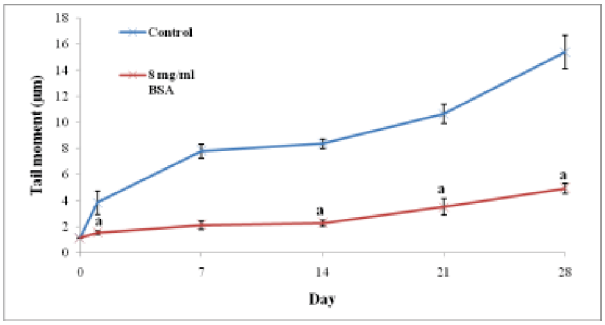
b.indicates significant compared to 1 mg/ml BSA group

c.indicates significant compared to 4 mg/ml BSA group

d.indicates significant compared to 8 mg/ml BSA group

e.indicates significant compared to 12 mg/ml BSA group

Figure 2.3 DNA integrity progression of bovine spermatozoa stored at 4 0C for 28 days. Results were presented in mean ± SEM, a indicates significantly different compared to control group, p<0.05.



4. Discussion

In the present study, we used three freezing-free preservation temperature (4, 25 and 37 0C) to find out optimum temperature for semen storage. Our results showed 4 0C optimally preseved semen viability throughout the assessment period. We postulated the presence of lower temperature could help in sparing energy for spermatozoa survival. Higher temperature aggressively induces higher cellular metabolism rates. This results in higher energy consumption that would lead to lower viability and shorter lifespan. This speculation is in agreement with our previous study. The tempearture of 4°C was identified as the best temperature compared to 25 0C and 37^{0}C for storage . Similar studies confirmed that 5 ^{0}C best preserved frozen-thawed whale spermatozoa compared with room temperature (20-25 ^{0}C) or 35 0C .

Increasing life span of stored spermatozoa at freezing-free temperature is important in semen preservation field. Supplementation of exogenous BSA seems to be able to improve the quality of the bovine spermatozoa. Our study has shown that BSA supplementation at the concentration of 8 mg/ml was able to improve viability percentage of stored spermatozoa. Based on past publications, we estimated that BSA had played a significant role in slowing down the rate of spermatozoa viability loss throughout the assessment period significantly. Our results were similar with previous study which pointed out that BSA improved viability of freeze-thawed spermatozoa .

We observed a sharp decline in spermatozoa viability in all groups after one day storage. This could be because of induction of cold shock. The high vulnerability of bovine spermatozoa to cold shock is related to the low membrane cholesterol to phospholipid ratio compared to spermatozoa from other species . Cold shock causes ultrastructural, biochemical, and functional changes resulting in spermatozoa immobility and death. Our speculation is in agreement with previous study by Yi et al. (2008). They proved that cold shock decreased the viability of cooled spermatozoa . Subsequently, at day seven until day 28 of assessment, we observed a stable progression in spermatozoa viability. Stabilizing phase might occur because of successful adaptation of spermatozoa to cool environment.

Cooling of spermatozoa at 4°C would decreases cellular metabolism through the decline in cellular energy production. Major energy pathway in cooled spermatozoa is glycolysis . So, the reduction of temperature to 4 °C would significantly decreased ATP production through the glycolysis pathway. Pyruvate is the most likely energy form during this condition. When surrounding temperature goes above 160C, the accumulated pyruvate is then quickly utilized to produce more energy. Since the spermatozoa's ATP substrate is unreplenishable, continuous consumption of energy sources through glycolysis will results in energy production decreases. This will lessens spermatozoa viability. Thus, addition of exogenous supply of BSA would likely improves the viability of preserved spermatozoa. BSA contains large quantities of fatty acid. Glycolysis used fatty acids to produce more and lasting energy compared to glucose. Previous research proved that BSA gives energy to spermatozoa, therefore improving spermatozoa survival rate following freezing and thawing procedure .

Cooling of spermatozoa produces free radicals. Free radicals causes oxidative damage and apoptosis. Supplementation of BSA at 8 mg/ml into preservation media improved spermatozoa viability through its antioxidant properties. This is in agreement with previous study in which supplementation of BSA into the media improved viability of the spermatozoa owing to its antioxidant properties .

Besides viability, integrity of spermatozoa DNA is important in determining fertilization. DNA damage has negative correlation with fertility outcomes . These have been attributed to the excessive amount of free radical surrounding the spermatozoa micro-environment. It has been demonstrated that cooling of spermatozoa at 4 0C increases production of ROS in a gradual manner against storage time . Previous researchers have identified the free radical contributors in the semen medium. They are releases of free radical from dying spermatozoa, leukocyte and endogenous leakage of electron from electron transport chain . The extrusion of cytoplasmic content during late spermatogenesis compound the problem because spermatozoa has a limited stores of endogenous antioxidant enzyme .

The buildup of free radical from various sources results in loss of motility and viability. Exogenous supplementation of antioxidant - as in this study BSA, confers spermatozoa with better protection against oxidative damage thus significantly improved semen quality compared to control for an assessment period of 28 days.

Endogenous antioxidant protection overwhelmed with multiple types and reactive free radicals produced. The depletion of the chemical scavengers allows free radical attack to biological materials nearby. Loss of motility, viability and increased DNA damage reflected a massive injury to DNA structure through oxidation and nitration reaction . As BSA is well known for its free-radical trapping properties, it is very likely that presence of extra BSA in media helped to scavenge various ROS and RNS . This conclusion is in line with a previous study. They showed that BSA reduced apoptotic rates and DNA damage in neuron cell .

They suggested that DNA protective effects were probably due to BSA antioxidant activity.

Another interesting finding from our study was that at lower (1 and 4 mg/ml) BSA concentration, the protein did not provide any sufficient protection to the spermatozoa's viability and DNA integrity. We speculated that at lower BSA concentration, BSA was not enough in giving enough energy supply and antioxidant protection to the stored spermatozoa. This would result in the early depletion of available energy source and lower energy supply for spermatozoa cellular metabolism eventually. On the other hand, our results indicated that at high BSA concentration (12 and 16 mg/ml), BSA seemed to have a harmful effect on spermatozoa viability and DNA integrity. We postulated that at high BSA concentration, the molecule tends to create a hyperosmotic condition, thus putting enormous stress on the spermatozoa membrane. The inability of the spermatozoa to withstand the hyper-osmotic condition is even more prominent when semen had just been thawed after being cryo-preserved . The inability to withstand the increased pressure could be due to the slight destruction of the spermatozoa's cytoskeleton membrane and/or disruption of the regulatory volume increase (RVI) system. In normal condition, RVI functions by offsetting hypertonicity condition. Unfortunately, at extreme hypertonic condition RVI tends to fail. We put forward that at 12 and 16 mg/ml BSA, RVI failed to work resulting in damaging effects to spermatozoa. Failure in RVI will further causes hypertonicity-independent apoptosis. The evidents favored involvement of both intrinsic (mitochondria) and extrinsic (death receptor) pathway activation . Low viability and motility seen in this study would likely due to the presence of large scale apoptosis of the current spermatozoa population.

Apart from apoptosis activation, we also believed that increased production of free radical in hypertonic condition might also contributed to lower viability percentage as well as DNA damage. Past studies indicated that hypertonic condition induced production of ROS . Our speculation is in line with Mahfouz, 2010 as well as De Iuliis et al., 2009, in which they demonstrated that increased ROS production had resulted in a significant reduction in viability and DNA integrity respectively .

Freezing-free presevation provides interesting alternative to spermatozoa preservation technique. However, short duration of storage is still a major problem and need more improvements. Our study indicated that optimum temperature for bovine storage was at 4 °C. Meanwhile, BSA supplementation at 8 mg/ml gave the optimum protection to spermatozoa viability as well as DNA integrity throughout the assessment period. The mechanism by which BSA protects cooled spermatozoa still needs to be elucidated. On top of that, the fertilizing competence of the spermatozoa preserved with BSA still needs to be confirmed to ensure that BSA is a potential molecule for freezing-free preservation agent.

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Conflict Of Interest

The authors declared of no conflict of interest exists.

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