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Effects of Alpha Lipoic Acids on Cattle Sperm Kinetics Using Computer Assisted Semen Analysis

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Abstract: This study investigated the protective effect of Alpha Lipoic Acid (ALA) on animal sperm quality using Computer Assisted Semen Analysis (CASA). Fresh semen sample collected from adult Limousin bulls. The experiment involved five test groups and a control. Alpha lipoic acid with different concentrations (0.1, 0.05, 0,025, 0.0125 and 0.00625 mmol mL^{-1}) incubated into semen from all test groups. They were cryopreserved and thawed after 1 h. CASA analysis prior to cryopresevation confirmed the baseline condition. While post-thawed investigation determined the changes in sperm quality between the various groups. CASA parameters used were percent motility, Average Path Velocity (VAP, μ sec^{-1}), Curvilinear Velocity (VCL, μ sec^{-1}), Straight Line Velocity (VSL, μ sec^{-1}), Amplitude of Lateral Head displacement (ALH, μ), Beat Cross Frequency (BCF, Hz), Linearity (LIN, ratio of VSL/VCL) and Straightness (STR, ratio of VSL/VAP). ALA statistically improved VAP, VCL, VSL, ALH and BCF particularly for ALA concentration > 0.05 mmol L^{-1}. On the other hand, it did not influenced LIN and STR. As a conclusion, ALA influences semicircular sperm motion and increases velocity. It might be useful as an additive in the extender or cryoprotectant agent to improve sperm motility.

Key words: Alpha lipoic acid, cattle, semen analysis, semen quality, sperm motility, Malaysia

INTRODUCTION

The global recession that began in 2008 and the unprecedented default or current rescue effort of many financial institutions has strongly affected the credibility of the international banking system. This in turn, indirectly damaged real economy. Developing countries, not fully integrated with international markets have been noted to be less affected. This unintended isolated will likely allow local microfinance institutions to continuously finance small scale projects during a time when foreign support to donor driven Non Government Organization (NGOs) is difficult to obtain.

Economic growth in Malaysia is largely driven by the manufacturing and service sectors. The emergence of the recession mentioned above has forced Malaysia to reevaluate its ability to generate growth. Since investment into Malaysia by United States and European countries has drastically reduced, expansion and diversification of the manufacturing sector has been severely affected (Furuoka, 2007). Ability to export finished goods has also been severely compromised. To minimize global recession impact and maintain growth, emphasis is now put on the further development of the agriculture sector (Wong, 2007). The intended development in this area includes increasing output of current agriculture products through biotechnology, improvement of distribution, development of alternative source of feeds and intensive farming utilizing existing infrastructure (Lokollo *et al*., 2006).

In Malaysia, traditional livestock farming is numerous but is done in small scale with a total animal of <20 cattle. A farmer will usually obtain calf sired by his only bull which is usually a Kedah-Kelantan species (K-K cattle). Due to absence of gene mixing, cattle quality of the farmer will usually be the same. To increase meat quality and improve ratio of silage to meat, introduction of a better bull species is required. The use of acclimatize Limousin bull instead of K-K cattle which are highly muscular is highly recommended. Since movement of a high performing bull from one area to another with intention to

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inseminate cattle in a particular region difficult, resource intensive and costly, an alternative method is required. To date, the best and cheapest method is the use of cryopreserved semen and mass Artificial Insemination (AI). Although, cryopreservation has been widely considered the best method for semen preservation and transportation, this technique does have its short comings. Not surprisingly, semen has been found to be extremely vulnerable to cold shock. The process of freezing and thawing the semen was also found detrimental to the spermatozoa due to the excessive generation of free radical. This is further exuberated by the high content of unsaturated fatty acids in the phospholipids of the plasma membrane and the relatively low antioxidant capacity of seminal plasma in some species of cattle (Jeong *et al*., 2009). Other complications include, retention of excess residual cytoplasm in the sperm midpiece and significant redox cycling of xenobiotics. In summary, the oxidative stress mentioned induces peroxidative damage in various parts of the sperm specifically the plasma membrane and DNA damage in both the mitochondrial and nuclear genomes (Aitken and Krause, 2001).

Due to these ramifications, a large percentage of sperm in thawed cryopreserved semen will have a significantly lower semen quality compared to fresh semen. Studies had indicated that compared to fresh, 8-fold times of cryopreserved bovine sperm were required to achieve equivalent fertilization rates *in vivo* (Bailey *et al*., 2000).

In order to minimize the oxidative stress during and after cryopreservation, introduction of antioxidant into seminal plasma or extender is essentially required. Past research has conclusively proven that since sperm viability are closely linked to its narrow physiological pH of 7.4-7.8, utilization of chemicals with strong acids that contain antioxidant properties may not provide protection bur rather cause mortality.

Recent studies indicated that incubation of semen with different concentrations of ascorbic acid 2-O-Alpha-Glucoside (AA-2G), improved motility and viability 1 and 3 h after incubation. Their ability to protect spermatozoa against the lipid peroxidation and the DNA damage have also been demonstrated conclusively (Yoshimoto *et al*., 2008). On the other hand, Alpha Lipoic Acids (ALA) had been reported to have additional functions by which they are able to regenerate vitamin C from reduced vitamin C in the presence of glutathione. This capability would allow more antioxidants to be present within the biological system without requiring increased vitamin C in the sperm media and thus shifting semen media to an acidic condition. Addition of ALA into the media does not cause a major shift of pH into acidic regions because the acid is categorized as a weak acid. Recent findings have indicated that ALA is able to enter the Krebs cycle, thus assisting in the production of ATP which is required in viable sperm. Based on this, a study was conducted to determine the effect of ALA on improving semen quality of cattle.

MATERIALS AND METHODS

All experiments were performed in compliance with institutional guidelines. Prior ethical approval was obtained. A total 15 semen straws from two (n = 2) adult Limousin-cross bred cattle with a constant 3 years production of quality sperm was pooled and used for this study. Inclusion criteria were the animals that were healthy, medication free and trained to give semen through an artificial vagina technique. Collected fresh semen was checked for quality by ensuring concentration of spermatozoa to be at least 20 billion mL^{-1} with at least 60% of the spermatozoa alive. Once, semen samples were certified as quality semen, samples were then mixed with an extender (Bioxcell®, France) at a ratio of 1:40, quarter by quarter to avoid sperm shock. The mixture was then allowed to incubate at 37°C for 3.5 h.

The semen was divided equally into 6 parts with triplicates in each part. Semen from part 1 was free from any intervention. Part 2-6 were then incubated in 0.1, 0.05, 0.025, 0.0125 and 0.00625 mmol mL^{-1}, ALA, respectively. All parts were then incubated for 1 h at 37°C. Sperm kinetic parameters that included percent motility, Average Path Velocity (VAP, μ sec^{-1}), Curvilinear Velocity (VCL, μ sec^{-1}), Straight Line Velocity (VSL, μ sec^{-1}), Amplitude of Lateral Head displacement (ALH, μ), Beat Cross Frequency (BCF, Hz), Linearity (LIN, ratio of VSL/VCL) and Straightness (STR, ratio of VSL/VAP) were later analyzed using Computer Assisted Semen Analysis (CASA) to ensure minimum inter-operator variation. Semen cryopreservation had involved leaving the semen samples in a 4°C cooler cabinet for 1 h to avoid cold-shock.

The samples were then loaded into a sperm straw with both ends plugged with Polyvinylpyrrolidone (PVP). Loading of the semen into the straws were all performed in a 4°C cooler cabinet. Later, the semen straws were then kept 5 cm above liquid nitrogen for 9 min. This step had produced a temperature of -70 to 96°C. Finally, the straws were then stored by immersing them into the liquid nitrogen (-196°C). All temperatures stated above were confirmed using a calibrated thermocouple.

An hour after a successful cryopreservation, samples were then taken out from liquid nitrogen and left at room

temperature for 5 min. Later, the samples were then put in warm water (37°C) for 2 min. Each ends of the straw were then cut and the thawed semen poured into individual test tube. The samples were then analyzed again by CASA. All data was then analyzed using correlation tests at significance level, $p<0.05$.

RESULTS

Motile and progressive showed significant results against ALA concentration (Table 1). CASA velocity parameters which were composed of percent Average Path Velocity (VAP, μ sec^{-1}), Curvilinear Velocity (VCL, μ sec^{-1}), Straight Line Velocity (VSL, μ sec^{-1}) were analyzed for statistical correlation. All parameters were found to have significant statistical correlation with the increase of ALA concentration (Table 2). On the other hand, CASA sperm movement parameters which were composed of Amplitude of Lateral Head displacement (ALH, μ), Beat Cross Frequency (BCF, Hz), Linearity (LIN, ratio of VSL/VCL) and Straightness (STR, ratio of VSL/VAP) shows mixed statistical correlation results (Table 3). LIN and STR were statistically insignificant while others were significant.

Table 1: Correlation values for sperm motility after incubated with alpha lipoic acids and grouped by motility status

Parameters	Pearson's correlation	p-values
Motile	-0.961	0.009*
Progressive	-0.940	0.018*

Negative correlation values indicate there is an inverse correlation between motility status and ALA concentrations. $p<0.005$ is considered as significant changes

Table 2: Correlation values for sperm velocity after incubated with alpha lipoic acids and grouped by velocity types (μm sec^{-1})

Parameters	Pearson's correlation	p-values
Average Path Velocity (VAP)	-0.957	0.011*
Straight Line Velocity (VSL)	-0.956	0.011*
Curvelinear Velocity (VCL)	-0.920	0.027*

VCL: Curvilinear Velocity; VAP: Average Path Velocity; VSL: Straight Line Velocity; Negative correlation values indicate that there is an inverse correlation between velocity type and ALA concentrations; $p<0.005$ is considered as significant changes

Table 3: Correlation values for sperm movement parameters after incubated with alpha lipoic acids and grouped by progression

Parameters	Pearson's correlation	p-values
Amplitude of Lateral Head displacement (ALH)	-0.770	0.127
Beat Cross Frequency (BCF)	-0.060	0.924
Straightness (STR)	0.932	0.021*
Linearity (LIN)	0.837	0.077

Negative correlation values indicate that there is an inverse correlation between parameters and ALA concentrations. While positive values indicate that the parameters have a linear correlation with ALA concentrations. STR is a ratio of VSL/VAP while LIN is a ratio of VSL/VCL; $p<0.005$ is considered as significant changes

Based on this result, it was concluded that LIN and STR effects were not dependent on the concentration of ALA in the extender. In general, detailed analysis of the result also clearly indicated that the increase of ALA into the extender improved the sperm's kinetic ability. The addition of ALA above the concentration of 0.05 mmol L^{-1} had profound effect on the sperm's form of movement. The sperms were found to be moving much faster forward but by taking a much curvier path. Head movement of the sperm had also increased significantly and this would explain the much curvier path taken by the sperm.

DISCUSSION

Spermatozoa plasma membrane contains high PUFA content. It is thought that it's primary function is to provide sperm with fluidity essential for fertilization (Wathes *et al*., 2007). The fluidity capability comes with a downside which is not well understood. Since, PUFA contains high amount of double bonds, it is highly susceptible to free radicals attack. When the membrane is continuously attacked, a distortion in the lateral and bilayer organization of lipids as well as the peroxidation of fatty acid moieties occurs. This would lead to membrane instability and sperm immobility eventually (Silva and Gadella, 2006). Addition of ALA is suspected to protect the fragile sperm membrane from radical attack by creating a chemical shield surrounding the mid-piece (aqueous layer) and within the structure itself (lipid layer). Surprisingly, result of motility from this study was different from the previous study on Boer buck sperm. In the latter study, Boer buck sperm motility had improved with addition of higher concentration of ALA. Unfortunately, concentration of above 0.025 mmol L^{-1} had reversed ALA protective effect and had now causes increased percentage of sperm immobility. On the other hand, a recent study showed that motility significantly increased when ALA at concentration of 0.05 mmol L^{-1} and above was added into the extender. It is suspected that this variability of results between species was attributed to the different specific tolerance to physiological pH range based on species and variability of membrane composition particularly the long-chain polyunsaturated fatty acid (Waterhouse *et al*., 2006).

Significant improvement of sperm overall velocity and partial movement parameters was likely due to the multiple function of ALA. It is suspected that general improvements of the motion of the sperms (ALH and BCF) were due to the direct comprehensive free radical

protection effect of ALA on the sperms membrane and extender. Ability of the ALA to regenerate vitamin C with the presence of glutathione and up-regulation of Glutathione-S-Transferase (GST) activity had further improved the extender's antioxidant capacity to withstand constant oxygen and/or nitrogen based free radical attacks. These protections had allowed the sperm to have a high fluidity membrane and retain structural integrity during cryopreservation and after thawing (Breininger *et al*., 2005).

Increase propulsion capability represented by VAP, VCL and VSL were likely due to the biochemical effects of ALA. When ALA was absorbed into the sperm, ALA molecules that were not involved with free radical scavenging would likely have been used to produce ATP through the Kreb's cycle in the mitochondria, GADPDs-glycolytic pathway present at the first 1/3 of the tail and the newly reassessed lactate metabolism pathway. Readily available ATP in these locations would then allow rapid contraction of myofibrils present in the tail (Gladden 2004). LIN and STR were not surprisingly insignificant, as VSL values had not increased as much as VAP and VCL. This phenomenon is thought to be beneficial to the sperm, as it would now allow sperm to move in a much more semicircular motion.

By moving in this fashion, sperm was able to swim further in a curved fallopian tube at a shorter time and thus allowing lower quantities of free radical being produced. This, in turn would lower the likelihood of the sperm DNA to be damaged by the free radicals thereby increasing the chances of successful fertilization (Lewis and Aitken, 2005). Despite a positive outcome in the result and the potential use of ALA as additional component in semen extender, further investigation on the effect of ALA towards the ability of the sperm to capacitate, fertilize and to achieve full term pregnancy are warranted (Tavares *et al*., 2008). Once confirmation on ALA ability in extender media has conclusively been proven, further research on the asthenozoospermia must be conducted.

CONCLUSION

Based on the results, researchers can conclude that addition of 0.05 mmol L^{-1} ALA and above in extender would produce a linear cryoprotective effect on the sperm during thawing and cryopreservation. The improvement of semen quality seen above not only were restricted to its percentage motility but also was extended to their kinetic characteristics.

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