

## BULL SPERMATOZOA MOTILITY: OPTIMIZATION OF COENZYME Q10 AND ALPHA-LIPOIC ACID CONCENTRATION

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### ABSTRACT

**INTRODUCTION:** Coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>) and alpha-lipoic acid (ALA) are antioxidants that play a role in ATP production and breakdown of free radicals. **OBJECTIVE:** The aim of this study was to optimize the concentration of CoQ<sub>10</sub> and ALA based on motility pattern of spermatozoa. **METHODS:** The pooled bull spermatozoa were initially sub-fractionated via electrophoretic separation which was performed at 20V for every two, four and six minutes. The sub-fractions were then further incubated with 0, 0.375, 0.75, 1.5, 3.0 and 6.0 μM of CoQ<sub>10</sub>. The other groups of sub-fractions were incubated with 0, 0.0625, 0.0125, 0.025, 0.05, 0.1 and 1.0 mmol/L of ALA. The incubation was done at 37°C for one hour. **RESULTS:** The effect of CoQ<sub>10</sub> was mostly seen on the sperm progression at every minute of separation. The only exception was sample incubated in 3 μM of CoQ<sub>10</sub> which shows significant increase on VCL over 0 μM and 6 μM of CoQ<sub>10</sub> at 4 minutes of separation. It was also noted that 1 mM of ALA mostly improved sperm velocity when the separation was collected at two and four minutes. Sperm population incubated with 1 mM of ALA also showed significant changes on STR and LIN at four minutes of separation. **CONCLUSION:** CoQ<sub>10</sub> and ALA influence sperm progression and velocity. This finding is important in the artificial reproductive techniques especially for in vitro fertilization (IVF). This will be further validated in future study.

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### [1] INTRODUCTION

A minimal amount of reactive oxygen species (ROS) exists in the male reproductive system under normal physiological circumstances. Studies have conclusively indicated that the presence of ROS in a small amount is essential to regulate various normal sperm functions. These includes capacitation, acrosome reaction, and sperm-oocyte fusion [1]. In the presence of immature germ cell, abnormal spermatozoa, leukocytospermia, cell debris and low antioxidant levels ROS production will be high. These conditions subsequently overcome the antioxidant level and promote a condition known as oxidative stress [2].

Oxidative stress has deleterious effects on the physiology of the spermatozoa such as lipid peroxidation, DNA damage and also has been associated with destruction of sperm motility [3]. The principal means of oxidative stress to impair sperm motility is by alteration of the membrane fluidity [4]. Alteration of the membrane fluidity happened mainly due to present of polyunsaturated fatty acid (PUFAs) in high concentration at the sperm membrane. This will cause the spermatozoa becomes

vulnerable to lipid peroxidation [5]. This situation is worsen by low concentration of scavenging enzyme in seminal plasma [6] and the inability of the intracellular antioxidant in providing protection to the outer layer of the membrane surrounding the head and tail of the spermatozoa [7]. Membrane fluidity plays a great role in regulating ion pump which include the ion pump that controls inwards and outwards movement of calcium ion into the spermatozoa. Alteration of the membrane fluidity will cause accumulation of calcium ion which consequently impaired sperm motility and eventually endanger the sperm survival [8].

As oxidative injury is evidently increased in male fertility, varieties of studies on antioxidant therapy have been conducted. These include study on glutathione [9], lycopene [10], vitamin C and vitamin E [11]. A number of studies also exist on the protective effects of coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>) [12] and ALA [13] towards male oxidative injury.

CoQ<sub>10</sub> is a fat-soluble molecule which is natural endogenously synthesized antioxidant in all humans and animals. CoQ<sub>10</sub> also

has the ability of being an antioxidant, enabled to quench the free radicals deteriorative effects and thus preventing lipid and protein peroxidation [14]. Moreover, CoQ<sub>10</sub> is also capable of reducing alpha-tocopheroxyl radical to alpha-tocopherol. This ability of eliminating pro-oxidant radical and regenerating vitamin E will render the hyper functioning of antioxidant in the spermatozoa environment. In addition to its antioxidant properties, CoQ<sub>10</sub> has been known to play an essential role in ATP production. It is supported by recent studies which had documented that CoQ<sub>10</sub> is concentrated in the midpiece region [15]. Hence, deficiency in CoQ<sub>10</sub> may give an indication as to why there is a reduction in sperm motility in some men. According to Lewin and Lavon, 1997, the CoQ<sub>10</sub> deficiency is particularly prevalent in men suffering from asthenozoospermia [16].

Another interesting compound known as alpha-lipoic acid (ALA) also can be found naturally in mitochondria. This compound is responsible as a coenzyme for pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase. Hence its essential for energy production [17]. In addition, ALA or its reduced form, dihydrolipoic acid (DHLA) can ameliorated the harmful effect of oxidative stress both in aqueous and membrane phases. Moreover, ALA and DHLA appear to have the ability to regenerate Vitamin C and thus indirectly recycles Vitamin E [18].

Based on these previous study, treatment of spermatozoa with ALA [13] and CoQ<sub>10</sub> [16] had indicated that there was improvement in sperm motility rate. However, there is only scanty information elaborating the effect of both antioxidants towards bull sperm motility. Furthermore, most of the previous study regarding the effect of antioxidant in improving male fertility problem had only evaluated on the percentage of motile sperm following treatment, improvement in sperm count, normal morphological sperm as well as the effect of the antioxidant treatment on the reproductive hormones [12]. In order to evaluate more detail on the effect of both antioxidants on sperm motility, we divided the parameter of the sperm motility into 2 categories which are sperm velocity (VCL, VSL, VAP;  $\mu\text{m/s}$ ) and sperm progression (WOB, LIN, STR; %).

Sperm velocity was the kinematics parameter which measure how fast of sperm movement from one position to the last position it has been detected. The curvilinear velocity (VCL) has been defined as the time-average velocity of a sperm head along its actual curvilinear path while straight line velocity (VSL) was the time-average velocity of a sperm head along its straight line between its first detected position and its last. On the same note, the average path velocity (VAP) was the time-average velocity of the sperm head along its average path. This path is computed by smoothing the actual path according to algorithms in the CASA instrument [19]. On the other hand, sperm progression was the pattern of sperm movement along their path. The wobble (WOB) parameter describe side to side movement of the sperm head, linearity (LIN) describe the path curvature and straightness of trajectory (STR) shows the average distance of the sperm from its origin on the average path during all frames analyzed.

Uniquely, we are also incorporating new sperm separation techniques known as electrophoretic separation system at which the sperm is separated at 20 V and is being isolated at 2, 4 and 6 minutes. The voltage being applied was based on the preliminary study done by our group where this voltage shows a good separation. While, isolation of the sperm at 2, 4 and 6 minutes gives a better recovery of motile sperm than isolation done at 8, 10 and 12 minutes of separation. The techniques applied in this study are implemented with slight modifications to the existing model of sperm electrophoretic separation proposed by Ainsworth et al. (2005) [20]. We prefer this sperm preparation technique as the outcome indicates a positive sign in improving the weakness of other previous sperm preparation. The previous sperm preparation technique which includes Percoll® gradient centrifugation, density gradient centrifugation, and swim-up technique has been associated with the production of ROS. Hereby, this study was conducted in order to optimize both antioxidant concentrations particularly on bull spermatozoa motility.

## [II] MATERIALS AND METHODS

### 2.1. Semen sampling

Multiple ejaculates from male adult bull were collected and pooled together. These were done by using artificial vagina at National Biotechnology Institute of Veterinary (IBVK), Jerantut, Pahang. The procedures followed have been reviewed by the institutional animal ethics research committee. Every ejaculation had yields up until 5 ml per ejaculate. Sperm motility were performed using CEROS (Hamilton Thorne Inc, Beverly, MA).

### 2.2. Semen preparation

About 1 ml from total semen sample was isolated for electrophoretic separations. For each electrophoretic separation, 60  $\mu\text{l}$  of sample were injected into the injection site. Electrophoretic separations were performed at constant voltage of 20 V for 6 minutes. For each 2 minute interval, 20  $\mu\text{l}$  of semen were taken out from the electrophoretic glass chamber. About 10  $\mu\text{l}$  was analyzed for sperm motility using Hamilton Thorne Motility-CEROS version 12.1c (Hamilton Thorne Inc, Beverly, MA), and the remaining 10  $\mu\text{l}$  were subjected to incubation with the selected antioxidant.

The series of concentration of CoQ<sub>10</sub> and ALA and the incubation time were done according to the previous study with slight modification [13,16]. The incubations were done by mixing 10  $\mu\text{l}$  of sperm with 10  $\mu\text{l}$  of ALA or CoQ<sub>10</sub> at different concentrations. Concentrations of CoQ<sub>10</sub> used in this study are 6.0, 3.0, 1.5, 0.75, 0.375 and 0  $\mu\text{M}$  while concentration for ALA are 1.0, 0.1, 0.05, 0.025, 0.0125, 0.0625 and 0 mmol/L. Incubation with ALA or CoQ<sub>10</sub> were carried out for 1-hour using 1.5 ml microcentrifuge tube at 37 °C. For ALA, serial dilutions were performed in 0.1 % DMSO while serial dilutions of CoQ<sub>10</sub> were carried out in Bioxcell® extender. Following 1-hour incubation, 10  $\mu\text{l}$  of sample was subjected to Hamilton Thorne Motility-CEROS version 12.1c (Hamilton Thorne Inc, Beverly, MA) to assess the sperm motility. The parameter of sperm motility were classified into 2 categories which are Velocity (VCL,  $\mu\text{m/s}$ ; VSL,  $\mu\text{m/s}$ ; and VAP,  $\mu\text{m/s}$ ) and Progression (WOB, %; LIN, %; and PROG, %).

### 2.3. Statistical analysis

The differences between concentrations were compared and results were expressed as mean ± SEM. Analysis of variance (ANOVA) using the SPSS software version 12.0.1 with Post-hoc test was performed to verify statistical significance. The p-values of <0.05 was considered as statistically significant.

## [III] RESULTS

### 3.1. Sperm kinematics

Following 1 hour incubation of the separated sample, the effect of CoQ10 and ALA were mostly seen on 2 and 4 minutes of separation [Table-1 and -2]. While only 0.375 µM of CoQ10 (p<0.05) gave a

significant effect in sperm population isolated at 6 minutes of separation particularly on WOB.

The effect of CoQ10 dominated sperm progression at every minutes of separation [Table-1, -2, and -3]. However, at 4 minutes of separation, 3 µM of CoQ10 (p<0.05) showed a significant changes on VCL over 0 µM and 6 µM of CoQ10 [Table-2].

Following 1 hour incubation of the separated sample with 1 mM of ALA caused significant changes mostly on sperm velocity isolated after 2 [Table-1] and 4 minutes [Table-2]. However, there was not much effect of ALA on sperm progression. The population incubated in 1 mM of ALA isolated at 4 minutes after separation [Table-2] which shows a significant changes on STR and LIN (p<0.05) were the only exception. The other series of ALA concentrations (0.025, 0.0125, 0.0625 and 0 mmol/L of ALA) were not demonstrated in the table. This was because spermatozoa incubated in these series of ALA concentration shows a very low motility and/or non-motile at all.

Parameter of motility	Concentration of CoQ10 (µM)						Concentration of ALA (mM)		
	0	0.375	0.75	1.5	3	6	0.05	0.1	1
Velocity (µm/s)	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
VAP	71.77±1.19	75.33±0.12	81.03±9.51	70.27±7.28	82.39±4.98	69.78±5.23	64.10±0.00	68.80±3.37	85.50±3.64 <sup>1,2</sup>
VSL	66.90±0.34	71.13±0.43	64.73±1.77	63.93±3.21	75.16±3.67	66.54±4.21	58.00±0.00	63.50±3.47	75.50±3.29 <sup>1,2</sup>
VCL	90.60±1.50	100.10±2.30	88.83±3.53	89.60±15.90	99.76±13.80	83.36±6.06	109.00±0.00	98.50±6.80	131.9±6.89 <sup>1</sup>
Progression (%)									
WOB	79.23±0.35	75.33±1.88	91.70±12.25	80.80±6.81	126.72±45.50	81.02±2.68	58.80±0.00	72.00±4.97	65.60±1.97 <sup>1</sup>
STR	93.40±1.05	94.43±0.52	82.17±9.84	92.03±5.01	91.90±1.25	95.90±1.01 <sup>c</sup>	90.60±0.00	92.40±2.16	88.30±0.73
LIN	75.67±0.78	74.67±2.33	75.33±0.88	76.33±9.83	71.89±3.46	78.60±2.72	57.00±0.00	67.20±5.63	59.20±1.97

**Table: 1. Effect of different concentration of CoQ<sub>10</sub> and ALA on sperm population isolated after 2 minutes of electrophoretic separation:** c= Concentration of CoQ<sub>10</sub> is significantly different compared to 0.75µM of CoQ<sub>10</sub> (p<0.05), 1= Concentration of ALA is significantly different compared to 0.05 µM of ALA (p<0.05), 2= Concentration of ALA is significantly different compared to 0.1 µM of ALA (p<0.05)

## [IV] DISCUSSION

Currently male fertility status is solely dependent on the semen quality evaluation. One of the most current important parameters used to evaluate the semen quality is sperm motility. Low number of motile sperm and abnormal sperm morphology are the common contributing problems in human as well as in animal exhibiting a fertility problem. Although the evaluation of semen quality could not fully rely upon sperm motility assessment, it has been well proven that immotile and/or poorly motile sperm will yield a very low fertilization rate. In certain circumstances, there is no fertilization of the oocyte unless advanced assisted reproduction techniques (ARTs) were used.

In this study, the most significant changes on sperm motility could be seen in sperm population isolated after 2 and 4 minutes of separation. There was not much effect of both antioxidants on sperm population isolated at 6 minutes. However, only 0.375 µM of CoQ10 (p<0.05) had showed a significant effect in sperm population isolated at 6 minutes particularly on WOB. The different effect seen in every population isolated at each minutes might probably be due to the differences in quality of the isolated sperm population. This isolation technique was based on sperm surface charged [20] and the surface charged is inevitably dependence on the integrity the sperm membrane [21]. The integrity of the sperm membrane might further influence the motility regulation of the spermatozoa. So, here we postulated that the sperm population isolated after 2 and 4 minutes of separation is the most desirable and in good quality rather than sperm population isolated after 6 minutes.

Parameter of motility	Concentration of CoQ10 (µM)						Concentration of ALA (mM)		
	0	0.375	0.75	1.5	3	6	0.05	0.1	1
Velocity (µm/s)	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
VAP	69.02±2.16	69.47±3.58	87.97±5.02	88.13±8.35	85.07±7.13	73.67±8.57	60.50±0.71	59.60±1.73	74.93±2.18 <sup>1,2</sup>
VSL	65.27±2.64	66.23±2.97	75.47±3.14	83.43±8.32	73.23±5.06	72.56±8.76	57.60±0.90	56.30±0.96	66.80±1.65 <sup>1,2</sup>
VCL	82.35±2.92	86.63±9.67	131.07±12.10	110.53±10.61	131.80±12.50 <sup>a,f</sup>	39.10±8.78	85.00±0.55	84.70±3.71	108.40±3.61 <sup>1,2</sup>
Progression (%)									
WOB	84.08±2.79	81.4±5.47	67.57±2.60 <sup>a</sup>	79.77±1.19	65.68±2.43 <sup>a,b,d</sup>	92.98±1.68 <sup>c,e</sup>	71.10±0.38	71.50±3.10	69.60±1.06
STR	94.53±2.44	95.47±1.13	86.00±2.25 <sup>a</sup>	94.57±0.80	87.06±2.33 <sup>a,b,d</sup>	85.41±12.50 <sup>c,e</sup>	95.00±0.40	94.70±1.26	89.70±1.12 <sup>1,2</sup>
LIN	80.83±4.02	80.00±5.29	61.33±4.09	78.00±2.08	59.50±3.29	91.23±1.86	69.60±0.22	67.70±2.88	63.30±1.04 <sup>2</sup>

**Table: 2. Effect of different concentration of CoQ<sub>10</sub> and ALA on sperm population isolated after 4 minutes of electrophoretic separation:** a= Concentration of CoQ<sub>10</sub> is significantly different compared to 0µM of CoQ<sub>10</sub> (p<0.05), b= Concentration of CoQ<sub>10</sub> is significantly different compared to 0.375µM of CoQ<sub>10</sub> (p<0.05),c= Concentration of CoQ<sub>10</sub> is significantly different compared to 0.75µM of CoQ<sub>10</sub> (p<0.05), d= Concentration of CoQ<sub>10</sub> is significantly different compared to 1.5µM of CoQ<sub>10</sub> (p<0.05),e= Concentration of CoQ<sub>10</sub> is significantly different compared to 3µM of CoQ<sub>10</sub> (p<0.05), f= Concentration of CoQ<sub>10</sub> is significantly different compared to 6µM of CoQ<sub>10</sub> (p<0.05),1= Concentration of ALA is significantly different compared to 0.05 µM of ALA (p<0.05), 2= Concentration of ALA is significantly different compared to 0.1 µM of ALA (p<0.05)

Parameter of motility	Concentration of CoQ10 (µM)						Concentration of ALA (mM)		
	0	0.375	0.75	1.5	3	6	0.05	0.1	1
Velocity (µm/s)	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
VAP	74.43±2.26	77.37±3.62	87.97±7.42	75.23±2.83	83.96±3.80	97.83±9.27	80.80±6.12	82.60±3.41	74.90±2.99
VSL	69.03±2.11	70.93±3.04	76.23±2.80	70.40±1.27	78.10±3.48	90.10±8.44	73.20±4.19	75.20±4.15	69.50±2.68
VCL	108.93±5.08	100.03±3.83	135.90±19.04	96.33±9.11	118.23±7.77	159.43±18.50	11.50±8.39	119.20±6.29	91.00±2.93
Progression (%)									
WOB	68.90±1.05	77.30±0.81 <sup>f</sup>	65.67±3.37	78.93±4.43	72.73±2.46	64.07±1.87	73.00±2.43	70.20±3.18	81.60±1.03
STR	92.73±0.42	91.70±1.13	87.37±4.44	93.70±1.83	93.13±0.80	92.30±0.20	91.70±1.05	90.60±1.62	93.10±0.79
LIN	66.67±0.82	73.33±0.66 <sup>f</sup>	61.33±6.69	77.00±4.93	69.43±2.84	61.00±1.86	67.50±2.84	66.00±3.62	76.40±1.25

**Table: 3. Effect of different concentration of CoQ<sub>10</sub> and ALA on sperm population isolated after 6 minutes of electrophoretic separation:** f= Concentration of CoQ<sub>10</sub> is significantly different compared to 6µM of CoQ<sub>10</sub> (p<0.05)

Following 1 hour incubation of the isolated spermatozoa, this study demonstrated that there were no significant changes on sperm velocity in sperm population incubated with CoQ<sub>10</sub>. The only exception was the population isolated at 4 minutes of separation. In this population, incubation with 3 µM of CoQ<sub>10</sub> gave a significant changes on VCL compared to 0 µM and 6 µM of CoQ<sub>10</sub> (p<0.05) [Table–2]. Based on these previous evidence, we believe that any CoQ<sub>10</sub> or ALA concentration which significantly affects VCL alone, is not suitable choice for conduction of ARTs especially IVF. According to Grippo et al. (1995) the increase in linearity and straightness of sperm movement are responsible for accelerated progressive movement.

These patterns of motility are essential by the spermatozoa to generate force in order to penetrate zona pellucida instead of quiescent state (VCL) of the spermatozoa prior to fertilization [22]. On the same note, Rodriguez-Miranda et al. (2008) showed that there was an increased in values for VSL and LIN in extracellular ATP (ATPe)-treated capacitated sperm. The success rates of IVF using the treated sperm were also increased [23]. Therefore, the spermatozoa that swim faster and straighter are more likely to give a great impact on IVF success rate and not the curve movement of the spermatozoa.

CoQ<sub>10</sub> at 6 μM concentration caused significant changes on sperm progression in each separation time. The mechanism by which CoQ<sub>10</sub> significantly affected the sperm progression is still to be determined. The possible explanation is the involvement of the CoQ<sub>10</sub> in the regulation of energy production as it is concentrated within the mitochondrial midpiece [15]. Since soluble adenylyl cyclase (sAC) which is vital for sperm motility activation is also confined to the midpiece region, it is possible for this highly lipophilic antioxidant to diffuse and directly protect the sAC. Subsequently it will influence the pattern of sperm progression.

On the other hand, the effect of ALA that observed primarily on sperm velocity might be due to the properties of ALA. ALA could have provided a shield throughout the sperm membrane. ALA also offers protection for both outer leaflet (aqueous layer) and the inner leaflet of the phospholipids (lipid layer). These properties might offer a wider fortification throughout the membranes of the midpiece and principal piece. In turn, the energy production is maintained and it may also retain membrane fluidity at the principal piece. Both energy production and membrane fluidity are vital for membrane-dependant regulation particularly for the regulation of motility. The latter would be critically important for the regulation of plasma membrane Ca<sup>2+</sup> channels, CatSper1 and CatSper2. This channels which are responsible for hyperactivated motility are confined to the principal piece [24]. Thus incubation of the sperm with ALA might directly accelerate the sperm velocity of the spermatozoa by influencing those channels present at the principle piece.

Based on the results we postulated that the particular effect of CoQ<sub>10</sub> and ALA on sperm progression and sperm velocity respectively is most probably due to the nature of the antioxidant properties itself and the different motility regulatory system presence in the different segments of the sperm. We have demonstrated that 6 μM of CoQ<sub>10</sub> shows a significant change on sperm progression in bull. In contrast 1 mM of ALA gives significant changes on the velocity of the sperm in bull. Both are crucial for improvement of sperm motility in bull population exhibiting the low motile sperm

## [V] CONCLUSION

In conclusion, both CoQ<sub>10</sub> and ALA may act as an ideal antioxidant for incubation of the sperm prior to ARTs. Incubation of spermatozoa with CoQ<sub>10</sub> will improve the pattern of sperm movement. On the other hand, incubation of spermatozoa with ALA gives a great impact on sperm velocity. However, little is known about the exact mechanism by which CoQ<sub>10</sub> and ALA influences the sperm progression and velocity of the sperm respectively. However, there are increasing interests to ascertain to what extent sperm motility would be affected if the concentrations of CoQ<sub>10</sub> and ALA are raised above 6 μM and 1 mM respectively. This could be determined through further research in the future.

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