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The effect of free fatty acids on the longevity of frozen thawed bull spermatozoa

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Abstract

Spermatozoa longevity is pivotal to successful fertilization as spermatozoa make their journey through the female reproductive tract. The quality of frozen-thawed bull semen is 50% lower than the fresh semen. The possibility that addition of fatty acids to sperm extender can improve semen quality is tested in the current study. To this end the effect of three fatty acids (oleic, palmitic and stearic acid) on frozen-thawed bulls semen was investigated. Thawed bull spermatozoa were incubated with different concentrations (0µM, 100µM, 250µM and 500µM) of each fatty acid and longevity (motility and viability) was estimated up to 6h. Total and progressive motility and viability was significantly improved after the supplementation of 500µM oleic acid, 250µM palmitic acid and 250µM stearic acid compared to the control condition. In conclusion, specific fatty acid supplementations can improve the longevity of frozen-thawed bull spermatozoa.

1. Introduction

Post-thaw quality of bull semen can be 50% lower than fresh semen (Pongsiri *et al.*, 2020) and this lower quality could also impact the longevity (motility and viability) of spermatozoa in vivo. In the female reproductive tract, spermatozoa have to travel a long distance to reach the oviduct where the oocyte will be fertilized. Possibly, the addition of fatty acids as metabolic substrates can improve the sperm longevity and this supplementation could be economical effective for improvement of artificial insemination companies. To this end the determination of the most effective fatty acid addition is needed for improving post-thaw bull semen longevity. Earlier studies demonstrated that addition of oleic, palmitic or stearic acid in sperm medium have improved sperm motility and viability of rooster and boar spermatozoa (Eslami *et al.*, 2016; Hossain *et al.*, 2007).

The biological significance of fatty acids is linked to their structural as well as their functional roles in cell membranes, maintaining membrane integrity and overall cell function. Cryopreservation and freeze-thaw cycles impact the cell membrane, therefore, a potential beneficial effect of fatty acid supplementation is of paramount importance for improving spermatozoal longevity. Fatty acids can not only improve membrane integrity, but also enhance the motility by serving as a substrate to generate ATP via mitochondrial β-oxidation (Zhu *et al.*, 2020a). Therefore, it is important to investigate the potential beneficial concentrations of fatty acids to improve the semen quality of frozen-thawed bull spermatozoa.

In the current study, it was investigated whether or not supplementation of free fatty acids could affect the longevity of frozen-thawed bull spermatozoa once they are incubated in a poor substrate medium that contains low levels (1 mM) pyruvate levels as only metabolic substrate. Furthermore, this poor medium may result in use of fatty acids for energy (as potential outcome) instead of a potential positive membrane change due to fatty acids.

2. Materials and methods

a. Experimental design

To investigate the influence of free fatty acids on longevity (motility and viability) of frozen-thawed bull spermatozoa, semen straws were thawed and Percoll® selected (for

procedure, see section below) spermatozoa were separately exposed to different concentration (0µM, 100 µM, 250µM and 500 µM) of oleic acid, palmitic acid or stearic acid, and incubated at 37 °C in a humidified incubator for 6 hours (h). Motility was assessed every hour up to 6h and viability was estimated at 1h, 3h and 6h incubation time.

b. Chemicals

All chemicals were purchased from Sigma Alderich (Zwijndrecht, The Netherlands), unless otherwise stated.

c. Frozen semen

Frozen semen of Holstein Friesian bulls was provided by CRV (Brédyk 32, 9088 BX Wirdum, The Netherlands) and semen was frozen in egg yolk based extender with a concentration of 54 million/mL in 0.25mL straws.

d. Semen processing

i. Preparation of Percoll® solutions

Percoll® 90% solution was prepared by diluting Percoll® stock (Sigma, Zwijndrecht, The Netherlands, GE 17-5445-02, 13.5mL) with D incomplete (10 times concentrated stock, 1.5mL). The composition of 10X D incomplete solution was as following: NaCl (0.054g/mL, Merck, Schiphol-Rijk, The Netherlands, 1.06404.1000), KCl (2.3mg/mL, Merck 4936), $NaHCO_3$ (0.021g/mL, Merck 1.06329.0500), Na_2HPO_4 (0.47mg/mL, Merck 6559), Na Lactate (0.0429mL of 60% solution, Sigma L 7900), HEPES (0.0238g/mL, Sigma H 6147), $MgCl_{2.6}H_2O$ (3.1mg/mL, Merck 5833) in LAL water (Cambrex, Wiesbaden, Germany, W50-500); pH 7.35 ± 0.05 and 280±2 osmolarity. Next, Percoll® 45% solution was prepared by diluting Percoll® 90% solution (5mL) with D incomplete (normal solution, 5mL). All solutions were stored at 4°C and prewarmed to 27°C prior to loading the density gradient.

ii. Semen thawing and density gradient centrifugation

Semen straws were thawed in a water bath at 37°C for 30 seconds and the contents of two semen straws were pooled together. Next, thawed semen was layered on top of Percoll® solutions in 15 mL falcon tube in the following order (bottom to top): 90% Percoll® Solution (1mL), 45% Percoll® Solution (1mL) and thawed semen (1mL, two 0.5mL straws). Subsequently, tubes were centrifuged at 700xG for 30 min at 27°C and sperm cells pelleted at the bottom of the tube. Next, the supernatant was pipped off (tubes tilted to 45° angle) leaving behind 150 µL, approximately, of the sperm pellet. Finally, sperm pellet was resuspended in 200 µL swim-up medium: 1.) 1.1 mg/mL of Na Pyruvate, Sigma P 2256; 3.) Pen/strep (10,000 units and 10,000 µg/mL, respectively), Gibco, Bleiswijk, The Netherlands, 15140-148; 4.) 6 mg/mL of BSA, VMP Biomed in 1X D incomplete.

iii. Semen treatment with fatty acids and incubation

After Percoll® treatment, four treatment groups were prepared for each fatty acid (oleic acid [C18:1], palmitic acid [C16:0] and stearic acid [C18:0]. Briefly, four 350 µL semen samples (each semen sample;150 µL semen pellet mixed with 200 µL of swim-up medium with different concentrations of each fatty acid; 0 µM (control), 100 µM, 250 µM and 500 µM, final concentrations in 350 µL of semen sample) were prepared and incubated in an incubator at 37°C with 95% humidity in the air (without CO_2) for 6

hours (incubation time; 1h, 2h, 3h, 4h, 5h and 6h) until analyses. Four semen samples of each fatty acid were analyzed at different incubation time (no separate semen sample was prepared for each time point).

e. Semen evaluation

i. Computer assisted sperm analysis

AndroVision® CASA system (Minitüb, Tiefenbach, Germany) fitted with a 20-X objective, a camera adapter (U-PMTVC, Olympus, Hamburg, Germany) and a camera with a resolution of 648 x 484 pixels (Pulnix TM-6760CL, JAI A/S, Glostrup, Denmark) was used for motility analysis. The CASA system was equipped with an automated stage. Following incubation (as mentioned above), semen samples (350µL) were incubated for 5 minutes at 38°C in a heating block before motility assessment. The microscope stage, glassware and pipette tips were pre-warmed at 38°C. After incubation, 3 µl of the semen sample was loaded in a chamber of pre-warmed Leja 4-chamber slide (chamber depth= 20µm, Leja Products B.V., Nieuw Vennep, The Netherlands) and motility parameters were assessed (at least 500 sperm cells in the center of the chamber). Sperm cell head recognition was set between 14 to 85 µm². Subsequently, following semen motility parameters were estimated; percentage of motile spermatozoa (total motility), percentage of progressively motile spermatozoa (progressive motility) and sperm kinematic parameters; average straight-line velocity (VSL), curved line velocity (VCL), average path velocity (VAP), linearity (LIN = VSL/VCL), straightness (STR = VSL/VAP), wobble (WOB = VAP/VCL), the average amplitude of lateral head-displacement (ALH) and beat cross frequency (BCF). The spermatozoa were considered to be motile when they met one of the following three criteria; 1) average head orientation change (AOC) higher than 7° and BCF greater than 25Hz, 2) DSL (distance straight line) greater than 3.5 µM and VSL greater than 8 µm/s and DSL>15 µm, 3) VAP greater than 15 µm. Spermatozoa were classified as progressively motile spermatozoa when one of the following criteria was met; 1) DSL>15 µm, 2) BCF>40 Hz and radius greater than 5 µm, 3) VSL>20 µm/s, 4) DCL (distance curve linear) ≥20 µm, 5) STR>0.3 and LIN>0.2, 6) Radius>10 µm, 7) BCF>30 and ALH>0.85.

ii. Sperm morphology

Viable and morphologically normal spermatozoa were counted by aniline blue/eosin staining (1:1 diluted stock solution; composition of stock solution: aniline blue, 0.1g/mL, Gurr 3400; eosin gelblich, 10mg/mL, Merck 15935 and 0.96 vol% of ethanol 96% in phosphate buffer solution ($Na_2HPO_4.2H_2O$ 100mg/mL, Merck, 6580; KH_2PO_4 3mg/mL, Merck, 4873; Gentamycin-sulphate 0.083 mg/ml in deionized water; pH 7.00 ± 0.05) at 1h, 3h and 6h incubation time for each concentration of oleic acid, palmitic acid and stearic acid. Briefly, a 5µL of semen sample was pipetted on a clean pre-warmed glass slide, followed by a drop of Aniline blue-eosin stain. After gently mixing the drops, another slide was used as spreader (45°angle) to make a smear. Following air dry or on a warming plate (37°C), viable (unstained) and morphologically normal spermatozoa were counted using an Olympus BX40 microscope (Olympus Optical Co., Tokyo, Japan) with 10X ocular and 100X oil immersion lens (1000-X magnification). A minimum of two viable and morphologically normal spermatozoa were counted.

3. *Statistical analysis*

Data were analyzed with general linear model for repeated measurements using SPSS (IBM, Version 29) and Bonferroni test was used for pairwise comparisons. Differences were considered significant when $p<0.05$.

4. *Results*

a. *Motility*

i. *Effect of oleic acid on motility*

Percentage of total motile spermatozoa was estimated over time (1h to 6h) following the supplementation of different concentrations of oleic acid (Figure 1). After 1h incubation, significantly higher total motility (57±6%) was observed after exposure to the 250 µM oleic acid concentration compared ($p=0.012$, $n=3$) to the total motility (40±3%) in control group (Figure 1). There were no differences in total motility after exposure to 100 µM, 250 µM and 500 µM of oleic acid during 1h incubation (Figure 1). After 2h incubation, the highest total motility (44±4%) was observed with 500 µM oleic acid and this was different than the total motility in control (20±10%, $p=0.004$) and 100 µM (27±2%, $p=0.034$) oleic acid group (Figure 1). There were no differences in the total motility between 250 µM (39±3%) and 500 µM oleic acid group after 2h incubation (Figure 1). After 3h incubation, although the total motility was higher in 250 µM (32±5%, $p=0.011$) and 500 µM (33±6%, $p=0.008$) oleic acid groups than control group (11±8%), there were no differences in comparison to 250 µM and 500 µM oleic groups (Figure 1a). After 4h incubation, the total motility was similar in the 250 µM (24±5%) and 500 µM oleic acid (24±5%) groups and was higher (than the control (6±6%, $p=0.012$ and 0.015 respectively) and 100 µM (9±3%, $p=0.030$ and 0.040 respectively) oleic acid groups (Figure 1). After 5h and 6h incubation, similar total motility patterns were observed (Figure 1): the total motility was higher ($p=0.007$ and 0.004 respectively) in the 500 µM (5h=21±9%, 6h=24±8%) oleic acid group than control (2±2%, 2±3%) and 100 µM oleic acid groups (6±2%, 3±3%, $p=0.030$ and $p=0.007$ respectively) after 5h and 6h incubation (Figure 1). Although the total motility was higher in the 500 µM oleic acid group after 5h and 6h incubation, this was not different than the 250 µM oleic acid group (14±3%, 10±4% [tendency, $p=0.052$], respectively, Figure 1).

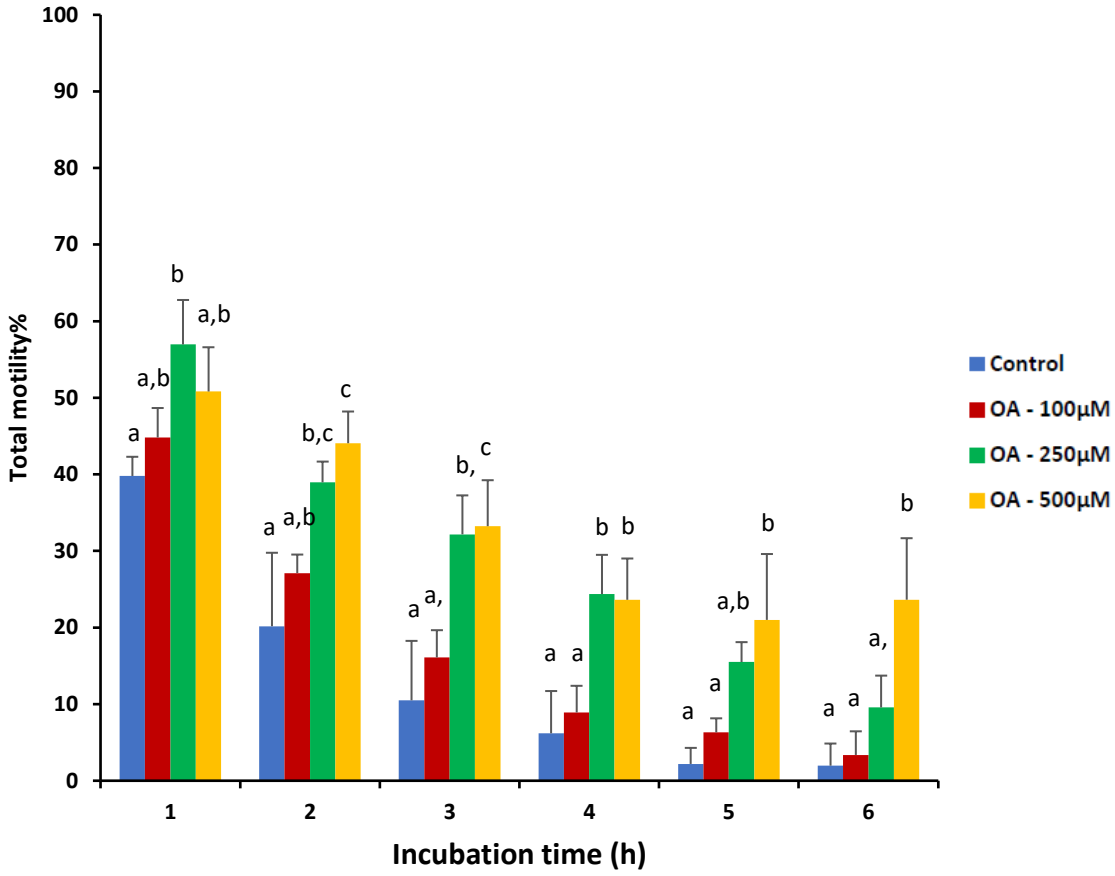

Figure 1.) Effect of different concentrations of oleic acid (OA) on total motility of frozen-thawed bull spermatozoa. Different superscripts above bars show significant difference between treatment group at an incubation time ($p<0.05$; n=3).

The percentage of progressively motile spermatozoa was estimated over incubation time of 1h to 6h after supplementation of different concentrations of oleic acid (Figure 2). After 1h incubation, the progressive motility was higher ($p=0.019$) in the 250 µM oleic acid group (49±4%) compared to the control group (32±4%). There were no differences between the 100 µM (37±4%), 250 µM (49±4%) and 500 µM (44±7%) oleic acid groups. After 2h, 3h and 4h incubation, the progressive motility was higher in the 250 µM and 500 µM oleic acid group (p=range, 0.006 to 0.022) than in the control group (Figure 2). After 5h incubation , the progressive motility was not different between the control (1±2%), 100 µM (3±2%) and 250 µM oleic groups (11±4%). There was a tendency for progressive motility in 500 µM oleic acid group (14±7%), than in the 100 µM oleic acid group (3±2%). At 6h incubation time, the progressive motility was higher in 500 µM oleic acid group (18±6%) than in the control (1±2%), 100µM (2±3%) and 250µM oleic acid group (6±4%) ($p=0.006$, $p=0.007$ and $p=0.033$ respectively).

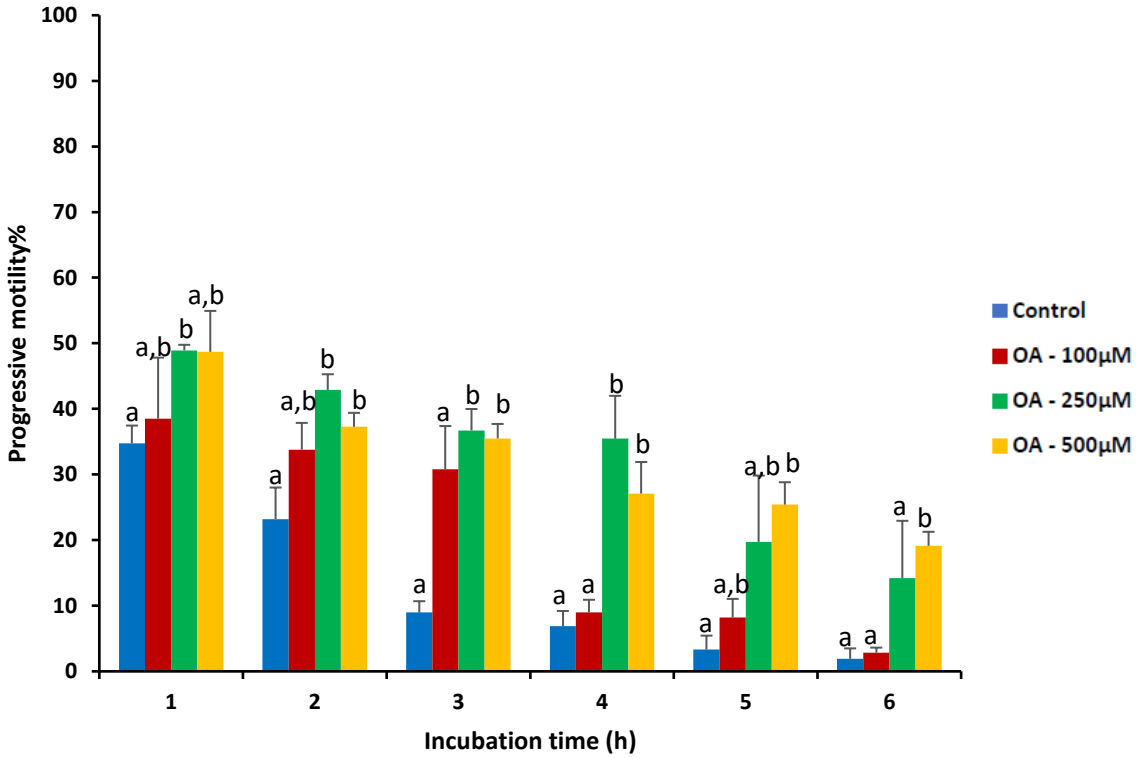

Figure 2.) Effect of different concentrations of oleic acid (OA) on progressive motility of frozen-thawed bull spermatozoa. Different superscripts above bars show significant difference between treatment group at an incubation time. ($p<0.05$; n=3).

ii. *Effect of palmitic acid on motility*

Percentage of total motile spermatozoa was estimated over time (1h to 6h) following the supplementation of different concentrations of palmitic acid (Figure 3). At 1h incubation time, there were no differences in total motility in all groups (Figure 3). At 2h incubation time, total motility was higher in 250 µM (47±5%) and 500 µM palmitic acid group (40±2%) than ($p=0.002$ and $p=0.027$ respectively)in the control (27±5%) group and there were no differences in total motility between 100 µM, 250 µM and 500 µM palmitic acid group (Figure 3). At 3h incubation time, total motility was higher in 250 µM palmitic acid group (39±4%) than ($p<0.05$) control (22±1%), 100 µM (26±2%) and 500 µM palmitic acid group (31±0.3%, Figure 3). At 4h and incubation time, total motility was higher in 250 µM (28±6%) and 500 µM palmitic acid groups (23±1%) than ($p=0.001$ and $p=0.008$ respectively) the control group (10±0.4%, (Figure 3). At 5h incubation time, there were no differences in total motility between 100 µM (11±1%), 250 µM (21±9%) and 500 µM palmitic acid groups (22±4%) (Figure 3). At 6h incubation time, although highest total motility was observed in 250µM (21±2%) palmitic acid group, it was not different than 500 µM palmitic acid group (12±6%) (Figure 3). Total motility was consistently higher in 250 µM palmitic acid group.

Percentage of progressively motile spermatozoa was estimated over time (1h to 6h) following the supplementation of different concentrations of palmitic acid (Figure 4). At 1h incubation time, progressive motility was higher in 250 µM palmitic acid group (49±5%) than ($p=0.034$, n=3) in the control (34±6%) group and there were no

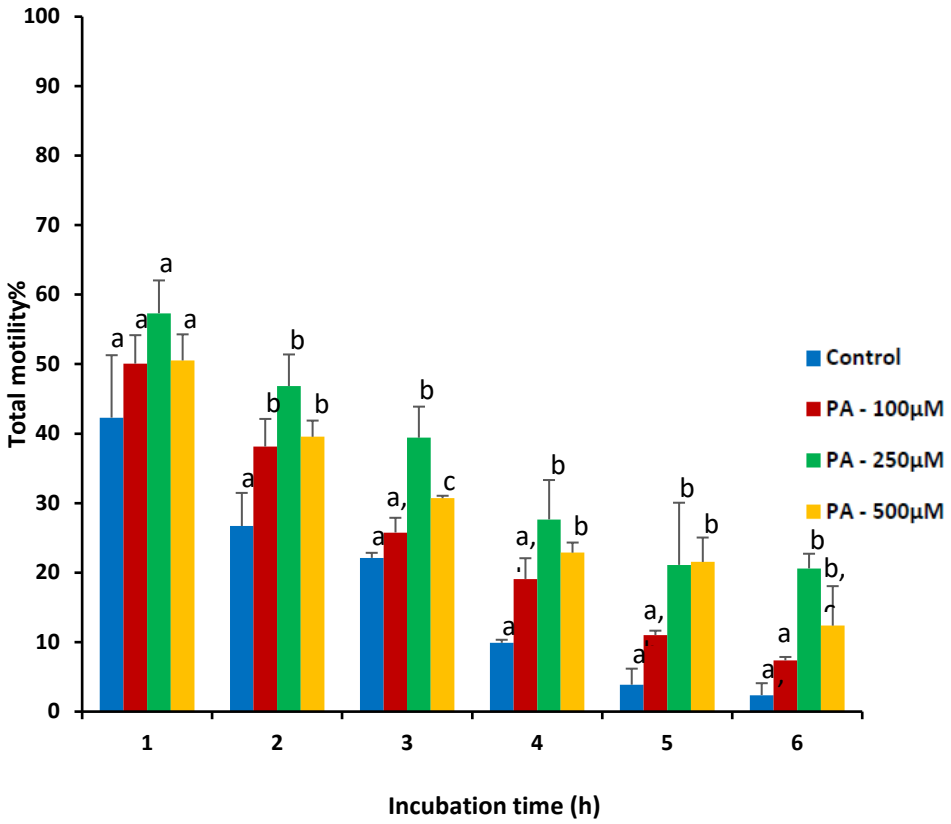

Figure 3.) Effect of different concentrations of palmitic (PA) acid on total motility of frozen-thawed bull spermatozoa. Different superscripts above bars show significant difference between treatment group at an incubation time. ($p<0.05$; n=3).

significant differences between palmitic acid groups (Figure 4). At 2h incubation time, progressive motility was lower in the control group (20±5%) than in the palmitic acid groups. At 3h incubation time, progressive motility was higher in 250 µM palmitic acid group (32±3%) than ($p=0.005$) in control (20±5%), 100µM (30±3%) and 500µM palmitic acid groups (33±1%). At 4h incubation time, progressive motility was higher in palmitic acid groups than ($p=0.043$) in the control group (4±2%, Figure 4). At 5h incubation time, progressive motility was higher in 250 µM (15±5%) and 500 µM palmitic acid groups (15±3%) than ($p=0.046$) in control (2±2%) and 100 µM palmitic acid group (5±2%). At 6h incubation time, although progressive motility was higher in 250 µM palmitic acid group (13±2%) than ($p=0.014$) in control and 100 µM palmitic acid group (3±1%), it was not different from the 500 µM palmitic acid group (8±5%).

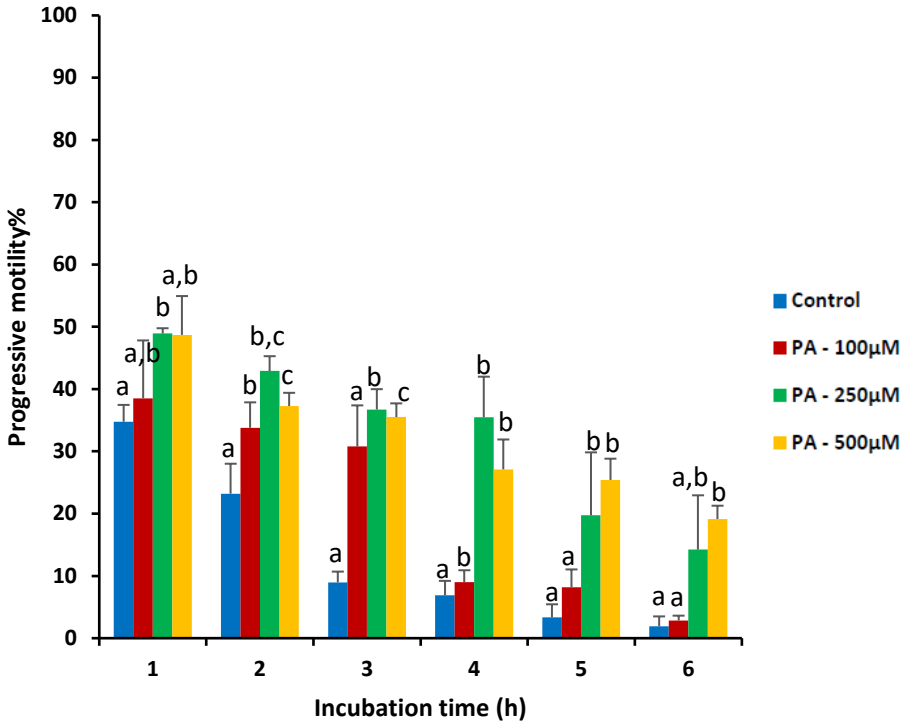

Figure 4.) Effect of different concentrations of palmitic (PA) acid on progressive motility of frozen-thawed bull spermatozoa. Different superscripts above bars show significant difference between treatment group at an incubation time. ($p<0.05$; $n=3$).

iii. *Effect of stearic acid on motility*

The percentage of total motile spermatozoa was estimated over time (1h to 6h) following the supplementation of different concentrations of Stearic acid (Figure 5). At 1h incubation time, there were no differences in total motility among groups (Figure 5). At 2h incubation time, total motility was higher in 250 µM (49±3%) and 500 µM stearic acid groups (45±4%) than ($p=0.007$ and 0.037 respectively) the control group (31±6%). There were no differences between control (31±6%) and 100 µM stearic acid group (40±5%) (Figure 5). At 3h incubation time, total motility was higher in 100 µM (38±11%), 250 µM (45±1%) and 500 µM stearic acid groups (43±4%) than ($p=0.004$) the control (13±2%) group (Figure 5). At 4h and 5h incubation time, total motility was higher in 250 µM (42±5%, 27±9%) and 500 µM stearic acid groups (34±4%, 33±3%) than ($p=0.006$) in the control (11±4%, 5±2%) and 100 µM stearic acid group (16±2%, 5±1%). Nevertheless, there were no differences between 250µM and 500µM stearic acid groups (Figure 5). At 6h incubation time, highest total motility was observed in 500µM stearic acid group (27±3%) and was higher than ($p=0.008$) control (3±2%) and 100µM stearic acid group (5±1%). There were no differences between 250µM (20±11%) and 500µM stearic acid groups (27±3%) (Figure 5).

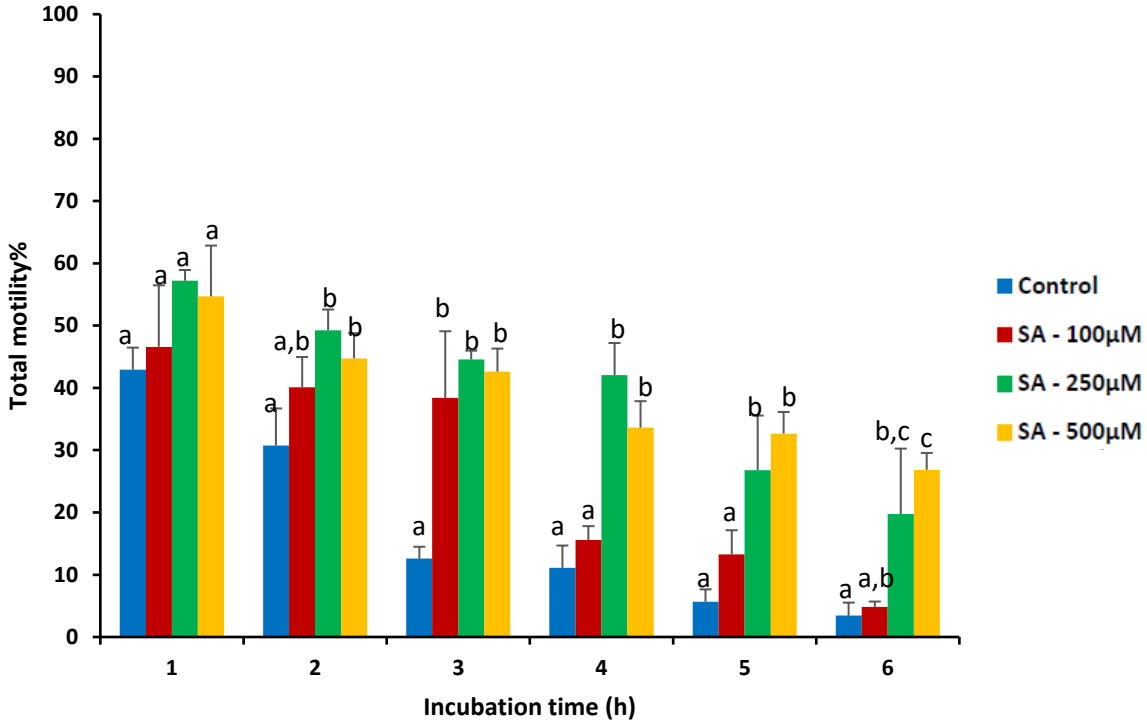

Figure 5.) Effect of different concentrations of stearic (SA) acid on total motility of frozen-thawed bull spermatozoa. Different superscripts above bars show significant difference between treatment group at an incubation time. ($p<0.05$; $n=3$).

The percentage of progressively motile spermatozoa was estimated over time (1h to 6h) following the supplementation of different concentrations of stearic acid (Figure 6). At 1h incubation time, there were no differences in progressive motility in control (35±3%) and 100 µM (39±9%), 250 µM (49±1%) and 500 µM stearic acid groups (49±6%). At 2h and 3h incubation times, progressive motility was similar in stearic acid groups and it was higher than ($p=0.038$) control group (Figure 6). At 4h incubation time, progressive motility was similar between 250 µM (35±7%) and 500 µM stearic acid groups (27±5%) and was higher than ($p=0.005$) in control (7±2%) and 100 µM stearic acid groups (9±1%). At 5h incubation time, higher progressive motility was observed in 500 µM stearic acid group (25±3%) than ($p=0.034$) in control (3±2%) and 100 µM stearic acid group (8±3%) (Figure 6). Nevertheless, there were no differences between 250 µM (20±10%) and 500 µM stearic acid (25±3%) (Figure 6). At 6h incubation time, although progressive motility was different in 500 µM stearic acid (19±2%) than ($p=0.014$) in control (2±2%) and 100 µM stearic acid groups (3±1%), it was not different than in 250µM (14±9%) stearic acid group (Figure 6).

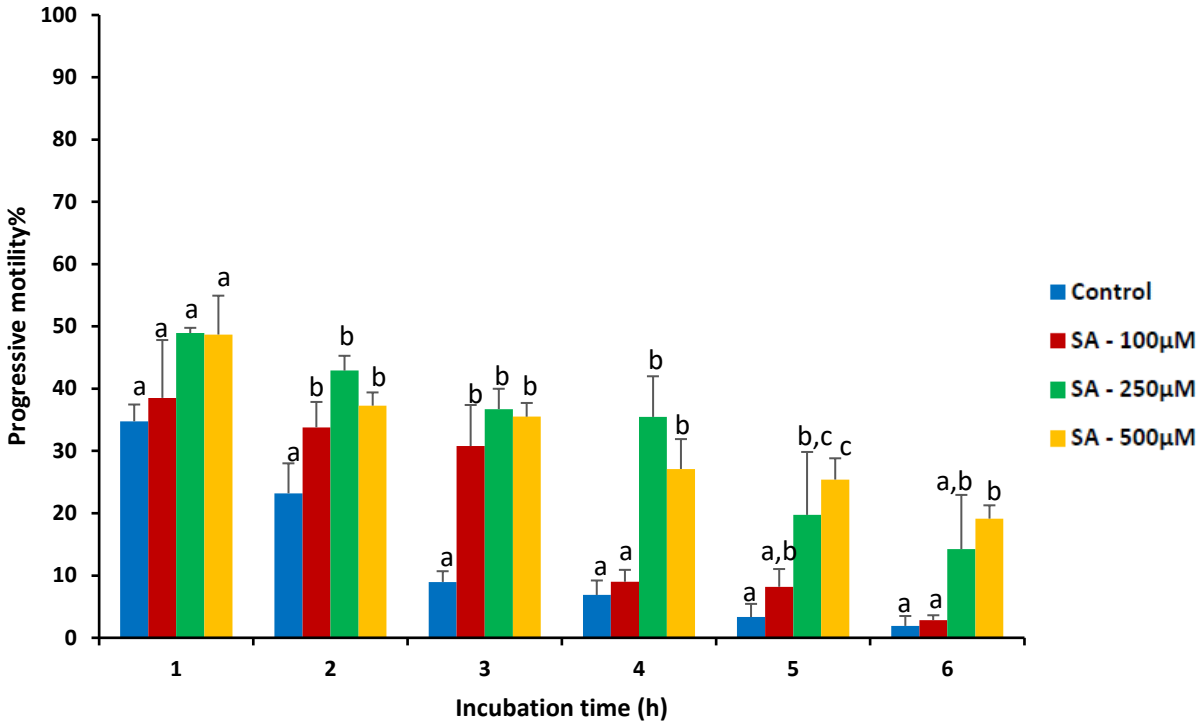

Figure 6.) Effect of different concentrations of stearic (SA) acid on progressive motility of frozen-thawed bull spermatozoa. Different superscripts above bars show significant difference between treatment group at an incubation time. ($p<0.05$; $n=3$).

b. *Comparison of fatty acids effect on motility and viability*

After initial analysis of motility data, 250 µM for palmitic acid and stearic acid, and 500 µM for oleic acid appeared to be the optimal concentrations to achieve higher total and progressive motility over time. Therefore, these three treatment groups, 250 µM palmitic acid, 250 µM stearic acid and 500 µM for oleic acid were compared to investigate differences in total motility, progressive motility and viability.

i. *Total and progressive motility*

A similar pattern of total and progressive motility was observed between the oleic acid, palmitic acid and stearic acid groups over time (Figure 7a and 7b respectively). Differences in total and progressive motility were only observed at 4h incubation time (Figure 7a and 7b respectively). Although total motility was higher in the stearic acid group (42±5%) than ($p=0.018$) in the oleic acid group (24±5%), it was not different from the palmitic acid group (28±6%). In contrast, progressive motility was higher in 250 µM (35±7%) stearic acid than ($p=0.024$) in 500 µM (19±4%) oleic acid and 250 µM palmitic acid groups (19±4%). There were no differences in total and progressive motility between three groups at 1h, 2h, 3h, 5h and 6h incubation times (Figure 7a and 7b).

ii. *Viability*

Percentage of viable spermatozoa was compared between 500 µM oleic acid, 250 µM palmitic acid and 250 µM stearic acid at 1h, 3h, and 6h incubation times. At 1h incubation time, there were no differences in percentage of viable spermatozoa between three treatment groups (Figure 7c). At 3h incubation time, percentage of viable spermatozoa was higher in 500 µM (35±4%) oleic acid and 250 µM (40±1%) stearic acid than ($p=0.026$) 250 µM palmitic acid group (27±2%). At 6h time, there were no differences in percentage of viable spermatozoa between three treatment groups (Figure 7c).

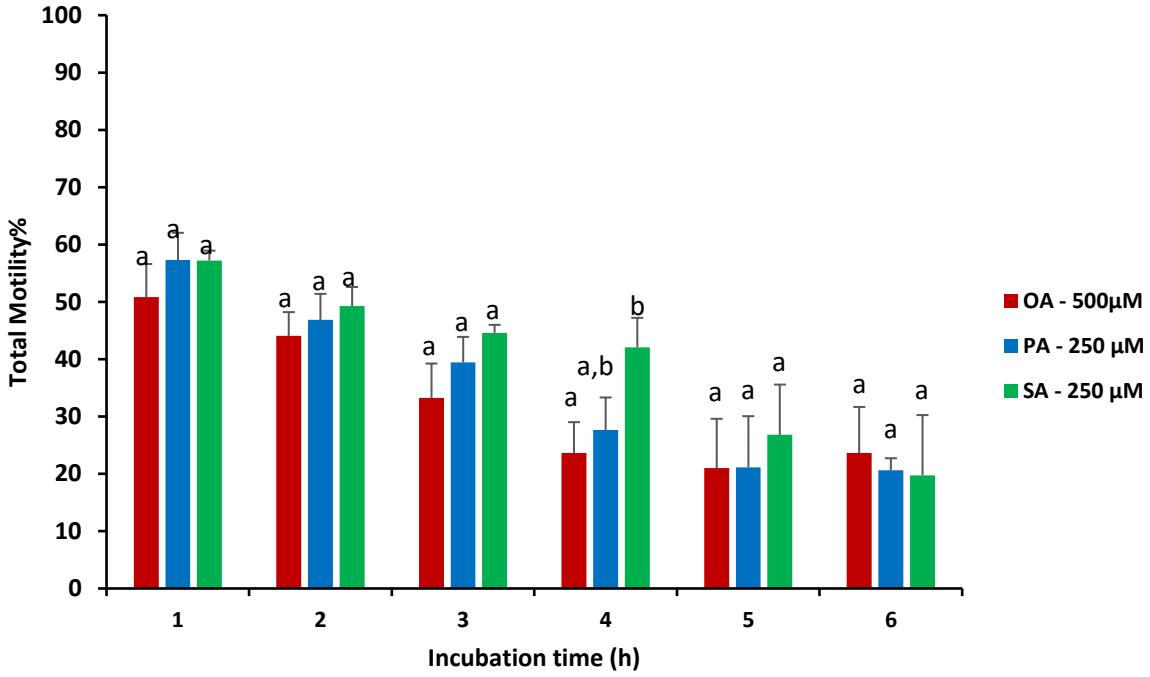

Figure 7a.) Comparison of the effect of oleic acid (OA), palmitic acid (PA) and Stearic acid (SA) on total motility of frozen-thawed bull spermatozoa. Different superscripts above bars show significant difference between treatment group at an incubation time. ($p<0.05$; $n=3$).

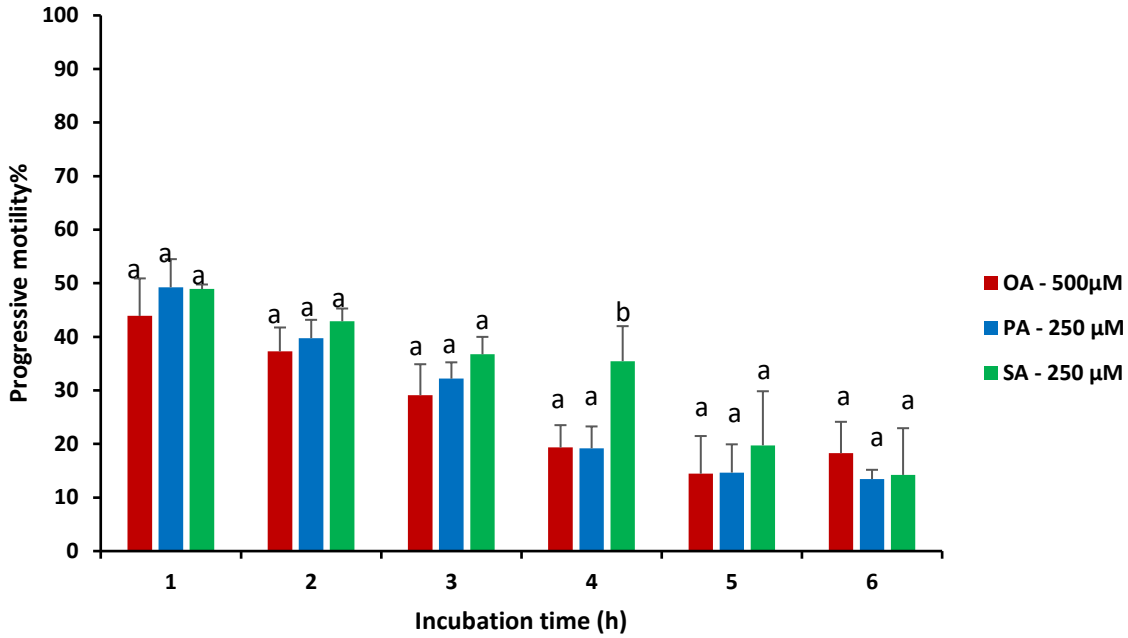

Figure 7b.) Comparison of the effect of oleic acid (OA), palmitic acid (PA) and stearic acid (SA) on progressive motility of frozen-thawed bull spermatozoa. Different superscripts above bars show significant difference between treatment group at an incubation time. ($p<0.05$; n=3).

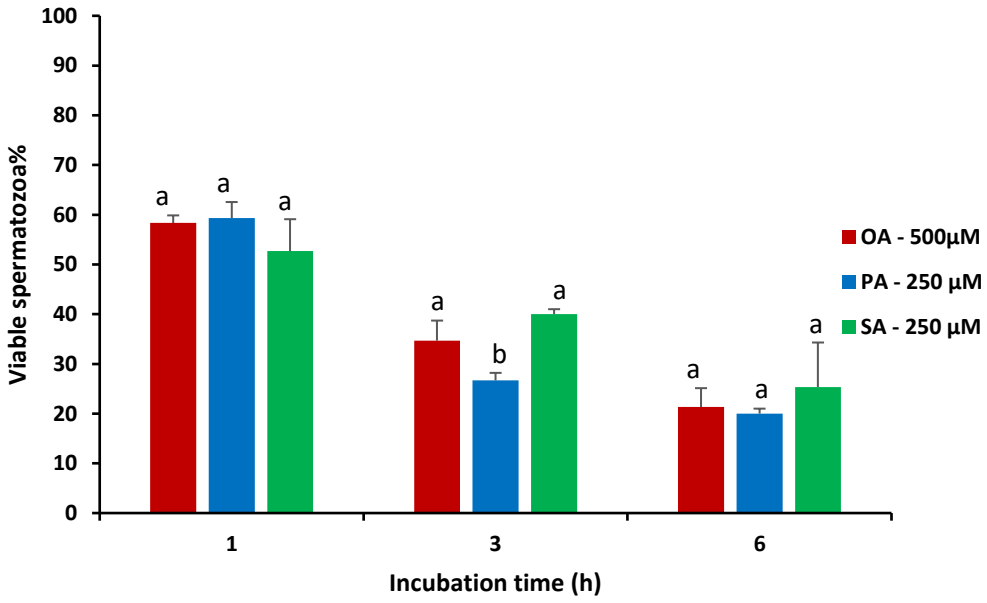

Figure c.) Comparison of the effect of oleic acid (OA), palmitic acid (PA) and stearic acid (SA) on viability of frozen-thawed bull spermatozoa. Different superscripts above bars show significant difference between treatment group at an incubation time. ($p<0.05$; n=3).

5. *Discussion*

In this study, the effect of different concentrations of three fatty acids (oleic acid, palmitic acid and stearic acid was investigated on the longevity (motility and viability) of frozen thawed bull semen. All doses of added fatty acids to the Swim-up medium

containing as sole other energy source 1 mM pyruvate resulted in improved longevity of frozen thawed bull sperm irrespective of the different fatty acid species (oleic acid, palmitic acid or stearic acid). Only stearic acid showed an additional increase in sperm longevity at 3h incubation time when compared to the oleic acid and palmitic acid groups.

Clearly, fatty acid supplementation improved the longevity, total and progressive motility of frozen-thawed bull spermatozoa in comparison to control group over time in a dose dependent manner. Oleic acid (500 µM), palmitic acid (250 µM) and stearic acid (250 µM) significantly improved the total and progressive motility of frozen-thawed bull spermatozoa in current study. These observations are in contrast with a previous study, where fatty acid supplementation did not affect the total and progressive motility of frozen-thawed bull spermatozoa (Vieira *et al.*, 2024). Previously, it has been shown that pig spermatozoa utilize fatty acids as an energy substrate via mitochondrial β-oxidation to generate ATP to maintain motility (Zhu *et al.*, 2020a). In the current study, bull sperm most probably used the fatty acid via mitochondrial β-oxidation (CoA formation via the carnitine transport system further oxidation of acetyl Co products of the beta oxidation in the TCA cycle) to generate ATP for longevity maintenance. Findings of the current study support this hypothesis as the control group with a low energy substrate condition resulted in poor longevity. Addition of fatty acids appeared to improve longevity of bull sperm.

In the current study, viability of frozen-thawed bull semen was well maintained up to 6h in all three fatty acid groups. Previously, fatty acid supplementation improved the viability of ram (Mortazavi *et al.*, 2020) and pig (Hossain *et al.*, 2007) spermatozoa. Palmitic acid supplementation has been shown to improve the viability of pig spermatozoa by maintaining the spermatozoal membrane integrity (Zhu *et al.*, 2020b). In the past, oleic acid addition in rooster semen extender resulted in an enhanced anti-oxidant activity and decreased lipid peroxidation (Huang *et al.*, 2016). In the current study, oxidative stress and lipid peroxidation were not investigated, but supplementation of fatty acids may have played a role in the maintenance of spermatozoal viability and motility of bull spermatozoa by preventing oxidative stress and lipid peroxidation. Future studies should investigate the potential of fatty acids to support spermatozoa in more detail.

6. Conclusion

Fatty acid supplementation of post-thawed bull spermatozoa improves the longevity (motility and viability) of bull spermatozoa.

7. References

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