Mansoura University

Faculty of Agriculture

Animal Production Department

APPROVAL SHEET

Title of Thesis: **Study on freezing buffalo semen using different antioxidants**

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SUMMARY AND CONCLUSSION

Five sexually mature buffalo bulls (4-5 years) raised at El-Gemmezah Animal Production Research Station were used in this study. Semen was collected from the experimental bulls for 4 weeks (40 ejaculates from all bulls).

Only semen with mass motility of 70% or more was pooled and divided into 7 replicates for different treatments. Semen was extended (1:10) using extender Tris-egg yolk (TEY), without supplementation (control), Leptin was supplemented to the extender at levels of (10,20 and 50 ng/ml) and Melatonin was supplemented to the extender at levels of (10^{-3}M, 10^{-6}M and 10^{-9}M) (7 group treatments).

Semen was equilibrated at 5^{0}c for 2,4,8 and 12 hours and storage at -196^{o}C for one month. Frozen straws for each extender treatments were thawed at rates 37°C/30 sec. Post-thawing recovery rate of motile, dead and abnormality of spermatozoa was calculated. Physical semen characteristics including motility, dead and abnormality of spermatozoa were determined in post- diluted, post-equilibrated (at different times) and post-thawed semen at thawing rate. Thereafter, reduction rate (%) in motile or rate of increase in dead and abnormal spermatozoa between dilution and equilibrium was calculated.

Activity of transaminases including aspartate (AST) and alanine (ALT) as well as activity of lactic dehydrogenase (LDH) were determined in post-freezing. Acrosome damage were used to evaluate the effect of different level antioxidant supplementation on quality of post-thawed spermatozoa at a rate of 37^{o}C/30 sec.

According to the obtained results for semen evaluation after thawing conception rate for buffalo cows artificially inseminated with semen extended

with (TEY) without antioxidant supplementation control, leptin (20 ng/ml) and melatonin (10^{-3}M).

The obtained results could be summarized as the following:

1. Experiment 1:

"Effect of different equilibration periods on some sperm characteristics in buffalo semen extended with Tris-egg yolk supplemented with different levels of leptin and melatonin"

1.1. Sperm characteristics:

1.1.1. Effect of equilibration period:

Progressive sperm motility percentage: It was the highest percentage overall mean motility with equilibration time (2h) (69.26%) and the lowest with equilibration time (12h) (57.55%), moderate with equilibration time (4h and 8h) (67.00 and 62.89%).

Dead sperm percentage: It was the lowest percentage overall mean dead sperm with equilibration time (2h) (25.43%) and the highest with equilibration time (12h) (39.12%), moderate with equilibration time (4h and 8h) (27.93 and 31.60%).

Sperm abnormality percentage: It was the lowest percentage overall mean sperm abnormality with equilibration time (2h) (18.73%) and the highest with equilibration time (12h) (26.10%), moderate with equilibration time (4h and 8h) (21.35 and 23.21%).

1.1.2. Effect of extender supplementation:

Progressive sperm motility percentage: Post- different equilibration time percentage overall mean motility was the highest ($P<0.05$) with LE (20 ng/ml) (73.62%), ranked the second with ME (10^{-3}M) (69.18%), moderate with

ME (10^{-6}M), LE (10 ng/ml), LE (50 ng/ml) and ME (10^{-9}M) (62.72, 62.46, 61.40 and 60.46%, respectively), while TEY (0) showed the lowest motility (59.37%).

Dead sperm percentage: Post- different equilibration time, dead sperm percentage overall mean was the lowest ($P<0.05$) for LE (20 ng/ml) and ME (10^{-3}M) (20.43 and 25.34 %) compared with the other types of treatments. It was moderate percentage with LE (10 ng/ml), ME (10^{-6}M), LE (50 ng/ml) and ME (10^{-9}M) (30.3, 33.3, 34.22 and 35.47%, respectively). It was the highest for TEY (0) without antioxidant supplementation (37.90 %).

Sperm abnormality percentage: Post- different equilibration time, sperm abnormality percentage overall mean was the lowest ($P<0.05$) for LE (20 ng/ml) and ME (10^{-3}M) (14.62 and 16.40%) compared with the other types of treatments. It was moderate percentage with LE (10 ng/ml), LE (50 ng/ml), ME (10^{-9}M) and ME (10^{-6}M) (22.59, 24.84, 25.56 and 25.90%, respectively). It was the highest for TEY (0) without antioxidant supplementation (26.53 %).

1.1.3. Effect of extender supplementation and equilibration period interaction:

The effect of extender supplementation interacted significantly ($P<0.001$, $P<0.01$ and $P<0.05$, respectively) on all sperm characteristics included progressive motility, dead and abnormal sperm percentages and these effects were reflected in similar trend of reduction in progressive sperm motility, dead and abnormal sperm percentages by increasing equilibration period. Progressive motility percentage was the highest, while dead and abnormal sperm percentages were the lowest in semen supplemented with 20 ng/ml leptin, followed by that supplemented with 10^{-3} M melatonin versus the lowest values in control un-supplemented semen.

2. Experiment 2:

"Effect of different levels of leptin and melatonin supplementations on sperm characteristics during freezing processes"

2.1. Sperm characteristics in post-diluted semen:

Progressive sperm motility percentage: Post-dilution progressive motility was the highest (P<0.05) with LE (20 ng/ml) (76.75%) and ME (10^{-3}M) (74.00), followed by LE (10 ng/ml), LE (50 ng/ml), ME (10^{-6}M) and ME(10^{-9}M) (70.0, 69.87, 69.75 and 69.12%, respectively), while TEY (0) showed the lowest motility (68.12%).

Dead sperm percentage: Post-dilution, LE (20 ng/ml) and ME (10^{-3}M) showed lower (P<0.05) Dead sperm percentage (17.00 and 19.50%) than that in the other treatments, while moderate with LE (10 ng/ml), LE (50 ng/ml), ME (10^{-6}M) and ME (10^{-9}M) (22.2, 25.00, 25.12 and 27.12%, respectively). The highest percentage of dead sperm was recorded for TEY (0) without supplementation (30.00 %).

Sperm abnormality percentage: Post-dilution, LE (20 ng/ml) and ME (10^{-3}M) showed lower (P<0.05) sperm abnormality (12.00 and 13.75%) than that in the other treatments, while moderate with LE (10 ng/ml), LE (50 ng/ml), ME (10^{-6}M) and ME(10^{-9}M) (17.37, 18.12, 18.37 and 19.25%, respectively). The highest percentage of abnormality was recorded for TEY (0) without supplementation (20.00 %).

2.2. Sperm characteristics in post-equilibrated semen:

Progressive sperm motility percentage: Post-equilibrated motility was the highest (P<0.05) with LE (20 ng/ml) (75.62%), ranked the second with ME (10^{-3}M) (71.25%), moderate with ME (10^{-6}M), LE (10 ng/ml), LE (50 ng/ml)

and ME(10^{-9}M) (66.00, 65.12, 64.25 and 63.75%, respectively), while TEY (0) showed the lowest motility (63.00%).

Dead sperm percentage: Post-equilibrium, dead sperm percentage was the lowest (P<0.05) for LE (20 ng/ml) and ME (10^{-3}M) (19.12 and 23.50 %) compared with the other types of treatments. It was moderate percentage with LE (10 ng/ml), ME (10^{-6}M), LE (50 ng/ml) and ME (10^{-9}M) (26.75, 29.12, 30.12 and 32.50%, respectively). It was the highest for TEY (0) without antioxidant supplementation (34.37 %).

Sperm abnormality percentage: Post-equilibrium, sperm abnormality percentage was the lowest (P<0.05) for LE (20 ng/ml) and ME (10^{-3}M)(14.12 and 16.00 %) compared with the other types of treatments. It was moderate percentage with LE (10 ng/ml), LE (50 ng/ml), ME (10^{-9}M) and ME (10^{-6}M) (21.25, 23.12, 24.62 and 25.37% respectively). It was the highest for TEY (0) without antioxidant supplementation (25.00 %).

2.3. Rate of change in sperm characteristics after equilibration:

Progressive sperm motility percentage: The reduction rate in motility between dilution and equilibrium was higher (P<0.05) LE (50 ng/ml) (7.98%), ME (10^{-9}M) (7.81%) and TEY (0) (7.54) and resulted in lowest (P<0.05) motility than the other types of treatments either the (1.47 and 3.69%) with LE (20 ng/ml) and ME (10^{-3}M),while moderate with LE (10 ng/ml) and ME (10^{-6}M) (6.97 and 5.42%).

Dead sperm percentage: The rate of increase in dead sperm percentage between dilution and equilibrium was higher (P<0.05) with ME (10^{-9}M), LE (50 ng/ml), ME (10^{-3}M) and LE (10 ng/ml) (22.54, 22.16, 21.87 and 20.42% respectively) and LE (20 ng/ml) (12.91%) resulted in lowest (P<0.05) dead

sperm than the other types of treatments either,while moderate with TEY (0) and ME (10^{-6}M) (14.76 and 17.74%).

Sperm abnormality percentage: The rate of increase in sperm abnormality percentage between dilution and equilibrium was higher (P<0.05) with ME (10^{-6}M) and ME (10^{-9}M) (40.18 and 30.26%). ME (10^{-3}M) and LE (20 ng/ml) (17.21 and 18.00%) resulted in lowest (P<0.05) sperm abnormality than the other types of treatments either,while moderate with LE (10 ng/ml),TEY (0) and LE (50 ng/ml) (22.64, 25.24 and 28.62% respectively).

2.4. Sperm characteristics in post-thawed semen:

Progressive sperm motility percentage: Post-thawing motility was the highest (P<0.05) LE (20 ng/ml) (59.25%), ME (10^{-3}M) (54.62%) and the lowest (41.25%) with TEY (0). LE (20 ng/ml) and ME (10^{-3}M) resulted in higher (P<0.05) motility than the other types of treatments either,while moderate with LE (10 ng/ml), ME (10^{-6}M), LE (50 ng/ml) and ME(10^{-9}M) (46.37, 45.00, 44.50 and 43.25%, respectively).

Dead sperm percentage: Post-thawing, dead sperm percentage was the highest (56.25%, P<0.05) with TEY (0) without antioxidant and the lowest (38.37 and 45.00%) for LE (20 ng/ml) and ME (10^{-3}M). while ME (10^{-6}M), LE (10 ng/ml), ME (10^{-9}M) and LE (50 ng/ml) showed the moderate values(49.62, 50.12, 51.12 and 52.73%, respectively).

Sperm abnormality percentage: Post-thawing, sperm abnormality percentage was the highest (41.25%, P<0.05) with TEY (0) without antioxidant and the lowest (20.00 and 23.12%) for LE (20 ng/ml) and ME (10^{-3}M). while LE (10 ng/ml), LE (50 ng/ml), ME (10^{-6}M) and ME (10^{-9}M) showed the moderate values(32.37, 36.62, 36.87 and 37.37%, respectively).

2.5. Recovery rate of sperm motility in post-thawed semen:

Progressive sperm motility percentage: Recovery rate (RR) of motile spermatozoa was the highest (P<0.05) LE (20 ng/ml) (77.22%), ME (10^{-3}M) (73.73%) and the lowest (60.41%) with TEY (0). LE (20 ng/ml) and ME (10^{-3}M) resulted in higher (P<0.05) motility than the other types of treatments either,while moderate with LE (10 ng/ml), ME (10^{-6}M), LE (50 ng/ml) and ME(10^{-9}M) (66.20, 64.46, 63.65 and 62.48%, respectively).

3. Experiment 3:

"Effect of different levels of leptin and melatonin on sperm acrosomal damage and enzyme activity in post-thawed semen"

3.1. Sperm acrosomal damage:

3.1.1 Effect of extender supplementation:

Acrosomal damage: Post-freezing, the percentage of the acrosomal damage of spermatozoa in post-thawed overall mean was the lowest (P<0.05) for LE (20 ng/ml) and ME (10^{-3}M) (9.62 and 11.87%) compared with the other types of treatments. It was moderate percentage with LE (10 ng/ml), ME (10^{-6}M), LE (50 ng/ml) and ME (10^{-9}M) (14.06, 14.93, 16.81 and 18.93%, respectively). It was the highest for TEY (0) without antioxidant supplementation (21.18 %).

3.1.2. Effect of incubation time:

It was the lowest percentage overall mean of the acrosomal damage of spermatozoa in post-thawed with incubation time (0h) (13.26%) and the highest with incubation time (2h) (17.42%).

3.1.3. Effect of interaction:

It was the higher percentage of acrosomal damage in all groups at 0 than after 2 h of incubation, being the lowest in semen supplemented with 20

ng/ml of leptin, followed by 10^{-3} M of melatonin and the highest in un-supplemented semen.

3.2. Enzyme activity in seminal plasma of post-thawed semen:

Activity of ALT: Post-freezing, ALT activity in post-thawed semen was the highest ($P<0.05$) for TEY(0) without antioxidant supplementation (59.25 U/l), moderate for LE (10 ng/ml), LE (50 ng/ml), ME (10^{-6}M) and ME (10^{-9}M) (45.75, 46.00, 46.50 and 46.50 U/l), and the lowest for LE (20 ng/ml) and ME (10^{-3}M) (37.75 and 43.25 U/l).

Activity of AST: Post-freezing, AST activity in post-thawed semen was the lowest for LE (20 ng/ml) and ME (10^{-3}M) (58.50 and 58.75 U/l), moderate for LE (10 ng/ml), LE (50 ng/ml), ME (10^{-6}M) and ME (10^{-9}M) (64.50, 64.75, 68.50 and 69.25 U/l), and the highest ($P<0.05$) for TEY(0) without antioxidant supplementation (98.75 U/l).

Activity of LDH: Post-freezing, LDH activity in post-thawed semen was the highest ($P<0.05$) for TEY(0) without antioxidant supplementation (392.50 U/l), moderate for LE (10 ng/ml), LE (50 ng/ml), ME (10^{-6}M) and ME (10^{-9}M) (350.50,351.75,355.50 and 356.75 U/l), and the lowest for LE (20 ng/ml) and ME (10^{-3}M) (316.25 and 330.50 U/l).

4. Experiment 4:

"Conception rate of buffalo cows artificially inseminated with semen supplemented with 20 ng/m of leptin, 10-3 M of melatonin compared with un-supplemented semen"

Conception rate: CR was higher for semen extended with antioxidant supplementation LE (20 ng/ml) and ME (10^{-3}M) (91.6 and 75.0%) as compared to that extended with TEY without antioxidant supplementation (66.6%).

CONCLUSION

Using artificial insemination is one of the most important tools in buffalo farms to accelerate genetic improvement (AI). AI plays a potential role in term of health and economic production and the most important need is high quality semen from a proven fertile bulls.

Leptin in the normal values has positive effects on male reproductive activity, but increasing leptin secretion from adipose tissue is known to have a deleterious effect on spermatogenesis and androgens secretion by Leydig cells ***(Tena-Sempere and Barreiro, 2002)****. If the concentration of melatonin in human semen is found to be lower than nanomolar range* ***(Bornman et al., 1989)****, there is high possibility that exogenous melatonin has some potential in correcting the influence of oxidative stress on human sperm.*

It is of interest to note that improving sperm characteristics by leptin (20 ng/ml) or melatonin (10^{-3} M), during freezing process was associated with marked reduction in sperm acrosomal damage and release of enzymes like AST, ALT and LDH, and reflected higher conception rate, significantly with leptin and insignificantly with melatonin in comparison with un-supplemented semen.

In conclusion, the obtained results revealed that adding 20 ng/ml of leptin and 10^{-3}M of melatonin to Tris-based extender had beneficial effects on motility, livability and normality of buffalo spermatozoa in diluted, equilibrated and thawed semen as compared to un-supplemented semen. These improvements were associated with increasing conception rate of buffalo cows which can be helpful for artificial insemination.