Effect of adding vitamin K with Carnitine to Tris egg yolk extender on buffalo abnormal sperm following cooling and cryopreservation

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Abstract

This Cryopreservation is important to preserve the semen for a long period for the purpose of spread the genetic materials on a large scale. However, 40-50% of buffalo beull sperm damaged following cryopreservation. The objective of the current research is to study the effect of adding different levels of vitamin K alone or carnitine alone or both to Tris extender on the abnormal sperm in male Iraqi buffaloes after treatment, cooling, and post-thaw. Four Iraqi buffalo was used in this study. Semen samples were collected weekly early at the morning. Samples were diluted with Tris egg-yolk extender. Following dilution, treatments were added to as following; T1=Control (no additives), T2= 0.03 ml vitamin K, T3= 7.5mMol Carnitine, and T4= 0.03 ml vitamin K+ 7.5mMol Carnitine. Following treatments, samples were cooled at 5 °C filled in 0.25 ml straw then freeze in liquid nitrogen (-196 °C). Two straws were thawed, two days following freezing, in water bath at 37 °C/ 30 seconds for evaluation. Total, head, midpiece, and tail sperm abnormalities were evaluated after three-time frame treatment, cooling, and post-thaw. The results showed that the head abnormalties in T1 was significantly greater compared to T3 following dilution and post-thaw, but it did not differ from T2, and T4. No significant differences bwteen treatments were found in the midpiece abnormalties for all the studied periods. Tail abnormalaties was signifcntly icreased in T1 compatred to T2 and T3. Total abnormalties following dilution, T2 and T3 decreased significantly compared to the T1. total abnormalties after cooling, T2 and T3 were significant decrease compared to T1 but did not differ from T4. For post-thaw, total abnormaties in T3 compared to T1, T2, and T4 showed a highly significant decrease. Regarding the effect of treatments over time, all studied parameters were significantly increreased overtime.In conclustion, supplementation of semen extders with vitamin K (0.03ml) or L-carnitine (7.5 mM) decreaseds the sperm abnormalties compared to control. More study is warrant to evaluate other sperm parameters on antioxidant and DNA fragmentation .

Keywords: vitamin K, carnitine, semen, cryopreservation, Iraqi buffalo, sperm abnormalities.

Introduction

Bull reproductive performance is one of the important factors which influence the reproductive efficiency of the herd. Male constitutes 50% of the herd's efficiency, which has a direct impact on the fertilization and pregnancy rate in buffaloes, which is

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reflected positively in increasing the economic return of livestock projects [1,2]. Artificial insemination technology is one of the most important assisted reproductive technologies in farm animals, which is used to spread significant genetic materials on a large scale, and the normality of cryopreserved semen is one of the basic signs of the success of this technology [3]. However, it was found that 40-50% of buffalo bull sperm are damaged during cryopreservation [4,5] due to thermal, chemical, osmotic, and mechanical stresses, which lead to a decrease in sperm motility and viability after liquefaction [6]. Improving the conditions for preserving buffalo semen is necessary to maintain sperm quality following cryopreservation. Recent works on buffalo and Holstien sperm quality were done with fat-soluble vitamins (K) (not published data) and water-soluble vitamin like amino acids (L-carnitine) [7], which found that the male abnormal sperm parameters were improved following vitamin K or L-carnitine additives compared to controls .

Vitamin K is one of the groups of fat-soluble vitamins (E, D, K, A) that are obtained from food because they cannot be manufactured in the body and which are characterized by a low toxic effect on animals compared to other fat-soluble vitamins [8–10]. It is worth noting that vitamin K plays an important role as an antioxidant in animal. The presence of these substances in low concentrations in natural conditions is necessary for physiological functions, which play an important role through their stimulation of many biochemical complexes in sperm [11–13]. It has been shown that low concentrations help in sperm maturation, adaptation, and hyperactivation of the acrosome interaction of sperm, and also contribute to the fusion of the egg [14]. But when it exceeds the equilibrium limit, it damages the cell wall through its sharing with a neighboring cell, which causes oxidation of fats in the cell wall sperm [15.[

Carnitine is a vital combination of two essential amino acids: methionine and lysine. It is produced in several organs in the body, including the liver, kidneys, and brain. It is a compound very similar to vitamins and is also found in large concentrations in the epididymis and sperm of mammals [16]. Therefore, it is believed that the sperm's acquisition of motility coincides with an increase in the concentration of carnitine [17]. The lack of carnitine in epididymal plasma led to a decrease and decline of the fertilizing ability of sperm [18]. In a study conducted by Lombardo and his colloquies, (2011), it was found that adding carnitine to semen improved the characteristics of sperm, such as individual motility and the integrity of the acrosome and plasma membrane, acting as an effective antioxidant [19]. The synergistic effect vitamins with vitamin like amino acids has not been studied yet. Therefore, we hypothesized that the synergistic effect of vitamin K and L-carnitine might enhance the abnormal sperm following treatment, cooling, and post-thaw. The objective of the current research is to study the effect of adding different levels of vitamin K alone or carnitine alone or both to Tris extender on the abnormal sperm in male Iraqi buffaloes after treatment, cooling, and post-thaw.

Material and Methods

Semen collection and evaluation :

The current research was conducted at Abo-Graib Artificial Insemination Center, Baghdad, Iraq. Iraqi buffalo local bulls (n=4) average age 5.5y; average weight 600 Kilogram. Samples were collected from all bulls by using artificial vagina. Briefly,

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artificial vagina was washed, sanitized, and dried one day before the collection day. Warm water (41-42 °C) was used to keep the artificial vagina warm to mimic the natural female conditions. At the day of collection, a teaser was used and falls mount was used before collection to increase the libido of the male. Semen was collected by graded tube kept in a warm jacket and transferred to the lab. transferred semen samples were kept in warm bath at 37 °C until evaluation. Knowing the evaluation will not exceed (7-10) mints. Semen was kept for 10 min in the water bath at 37°C before being evaluated for its quality and suitability for extension and preservation. Samples were evaluated for viability under microscope to make sure the samples are viable. All semen samples, after testing, were pooled to reduce the individual make effect. Aliquots were spliced into two replicates (4 ml) for further processing. Semen samples with concentration greater than 250 X106 sperm/ml, and greater than 40% individual motility were considered for evaluation. Ate the morning, one sample per week was collected from each bull for five weeks.

Semen processing

Tris egg yolk extender was prepared one day before collection then semen were added to the extender. Pooled semen was aliquot into four tubes each one considers a group. Treatments were added to the samples as following; T1=Control (no additives), T2= 0.03 ml vitamin K, T3= 7.5mMol Carnitine, and T4= 0.03 ml vitamin K+ 7.5mMol Carnitine. Following treatments, samples were cooled gradually to 5 °C for 1.5 hours for adaptation. After reaching the mentioned temperature, the samples were left for up to 4 hours. After cooling, slow freezing process was used following semen after filled in 0.25 ml straw (IMV Technology, France). Briefly, straws were interred to a special the liquid nitrogen tank and straws were kept on a aluminum holder to make sure that the straw was above the liquid about 5 cm (-120 °C) for 9 minutes. After that, straws were immersed into liquid nitrogen (-196 °C) and kept in storage tank until evaluation after 2 days [20]. At the time of evaluation, straw was thawed in water bath at 37 °C/ 30 seconds. For evaluation, two straws were thawed and the first drop was neglected and the second drop was evaluated. Total, head, midpiece, and tail sperm abnormalities were evaluated after three-time frame treatment, cooling, and post-thaw .

Abnormal sperm assessment

The percentage of abnormal sperm slide was prepared according to the method described by [21]. Two drops of Esoin-Nigrosin stain were added to a warm glass slide. A drop of semen sample was added at the stain then smear was done then tested under microscope 40x magnification (Por-Way Hb, Japan). Abnormal sperms were calculated according to [22]. 200 abnormal sperm were counted under a microscope at 40× magnification. The abnormalities were classified according to [23]. Head abnormalities was classified into Pyriform, Dwarf, Narrow, Giant, Twin, and Detached. Regarding the midpiece abnormalities were classified into Swollen, Twin, Protoplasmic droplet. The tail abnormalities were classified into Coiled, Bent, and Twin. The percentage of abnormal sperm was calculated according to the equation below :

$$\text{Abnormal sperm } \% = \frac{no.abnormal\ sperm}{Total\ no.of\ sperm} X100$$

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Statistical analysis

Onw way analysis of variance repeated measures was used to determine the effect of treatments over time on buffalo abnormal sperms with statistical analysis system [24]. Duncan multiple range test was used to show the differences between means [25.[

Results and Discussion

Effect of adding vitamin K or L-carnitine or both to the Tris-egg yolk extender in Iraqi buffalo bulls' semen on sperm head abnormalities :

In the current study, the statstical analysis showed that there were a significant differences between treatments in head abnormalties following dilution, cooling, and post-thaw (Figure 1). The head abnormalties in T1 was significantly greater (P<0.05) compared to T3 following dilution and post-thaw, but it did not differ from T2, and T4. However, the head abnormatlites did not differed between treatment follwing cooling at 5 °Cperiod (Figure 1). Regarding the effect of treatments over time, there were a highly significant differences (P<0.01) found between the period after dilution, cooling, and post-thaw. In T1 and T4, head abnormalties were significantly increased (P<0.01) post-thaw. However, there were no significant differences in head abnormalties in the following dilution and colling (Figure 1). In addtion, T2 and T3 were significantly increased (P<0.01) post-thaw compared to the post dilution (Figure 1.(

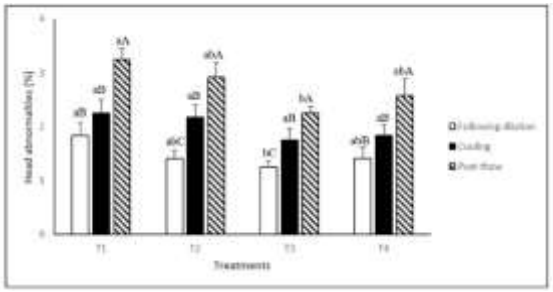

Figure 1. Effect of adding vitamin K or L-carnitine or both to the Tris-egg yolk extender in Iraqi buffalo bulls' semen on sperm head abnormalities. Different lowercase letters were significantly differed (P≤0.05) between groups within the same time of evaluation. Different uppercase letters were significantly differed (P≤0.01) between times of evaluation within the same group. The sperm assessment was evaluated following dilution, cooling, and post-thaw of adding vitamin K or L-carnitine or both (T1= control (no treatment), T2=0.03 ml vitamin K, T3=7.5 mM L-carnitine, and T4=0.03ml vitamin K and 7.5mM L-carnitine.(

Effect of adding vitamin K or L-carnitine or both to the Tris-egg yolk extender in Iraqi buffalo bulls' semen on sperm midpiece abnormalities:

The results showed that there were no significant differences bwteen treatments in the midpiece abnormalties for all the studied periods (Figure 2). However, the effect of treatments overtime was significatly increassed overtime within treatment. In the post-thaw period were significantly increased

(P<0.05) compared to following dilution but did not differ from post cooling (Figure 2.(

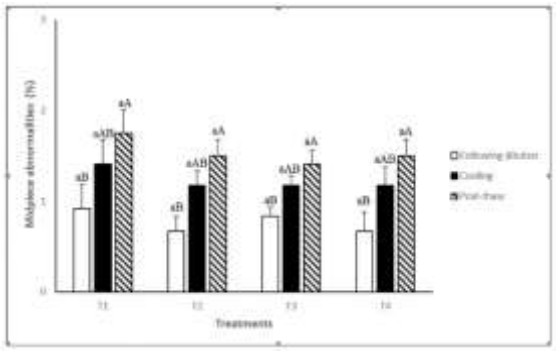

Figure 2. Effect of adding vitamin K or L-carnitine or both to the Tris-egg yolk extender in Iraqi buffalo bulls' semen on sperm midpiece abnormalities. Different lowercase letters were not significantly differed between groups within the same time of evaluation. Different uppercase letters were significantly differed (P≤0.01) between times of evaluation within the same group. The sperm assessment was evaluated following dilution, cooling, and post-thaw of adding vitamin K or L-carnitine or both (T1= control (no treatment), T2=0.03 ml vitamin K, T3=7.5 mM L-carnitine, and T4=0.03ml vitamin K and 7.5mM L-carnitine.(

Effect of adding vitamin K or L-carnitine or both to the Tris-egg yolk extender in Iraqi buffalo bulls' semen on sperm tail abnormalities:

Our results showed that there was a significant differneces (P<0.05) between treatments in the tail abnormalties follwing cooling. Tail abnormalaties was signifcntly icreased in T1 compatred to T2 and T3 but did not differed from T4 (Figure 3). However, the results showed that there were no significant difference between treatments in the tail abnormalties following dilution and post-thaw. Regarding the effect of treatment overtime, the tail abnormalties were significantly increased (P<0.01) in the post-thaw compated to following dilution and cooling wihtin T2, T3, and T4 but did not differ in

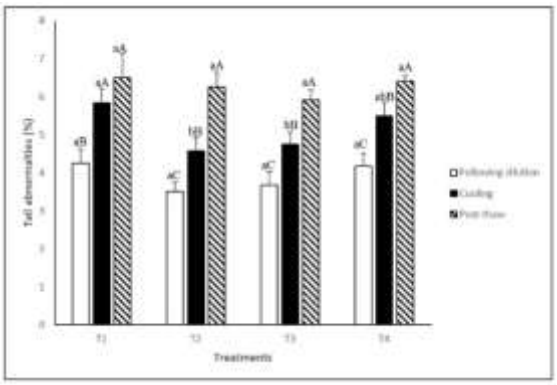

Figure 3. Effect of adding vitamin K or L-carnitine or both to the Tris-egg yolk extender in Iraqi buffalo bulls' semen on sperm tail abnormalities. Different lowercase letters were significantly differed between groups within the same time of evaluation. Different

uppercase letters were significantly differed (P≤0.01) between times of evaluation within the same group. The sperm assessment was evaluated following dilution, cooling, and post-thaw of adding vitamin K or L-carnitine or both (T1= control (no treatment), T2=0.03 ml vitamin K, T3=7.5 mM L-carnitine, and T4=0.03ml vitamin K and 7.5mM L-carnitine(

Effect of adding vitamin K or L-carnitine or both to the Tris-egg yolk extender in Iraqi buffalo bulls' semen on sperm total abnormalities:

The results of Figure 4 showed that there were a significant differences (P<0.05) in the percentage of total abnormalties between the treatments (Figure 4). The T2 and T3 decreased significantly compared to the T1 in the total abnormalties following dilution but did not differ from T4. The results also showed a highly significant decrease (P<0.01) in the percentage of total abnormalties after cooling at 5°C for T2 and T3, respectively, compared to T1 but did not differ from T4 (Figure 4). The results also showed a highly significant decrease (P<0.01) in the percentage of total deformations post-thaw T3 compared to treatments T1, T2, and T4. Regarding the percentage of total abnormalties over time, the results showed that there were a significant differences in the total deformities within treatments. In treatment T1, T2, T3, and T4, the total abnormalties increased significantly (P<0.01) post-thaw and comapred to following cooling and dilution (Figure 4.(

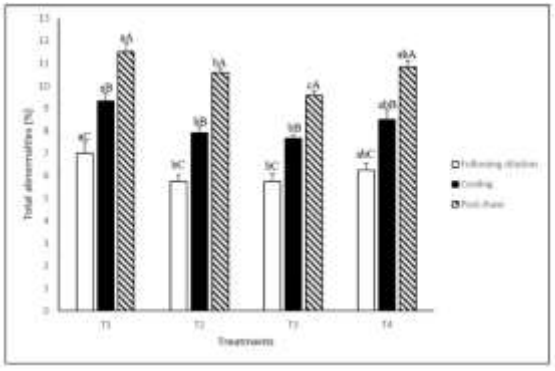

Figure 4. Effect of adding vitamin K or L-carnitine or both to the Tris-egg yolk extender in Iraqi buffalo bulls' semen on sperm total abnormalities. Different lowercase letters were significantly differed between groups within the same time of evaluation. Different uppercase letters were significantly differed (P≤0.01) between times of evaluation within the same group. The sperm assessment was evaluated following dilution, cooling, and post-thaw of adding vitamin K or L-carnitine or both (T1= control (no treatment), T2=0.03 ml vitamin K, T3=7.5 mM L-carnitine, and T4=0.03ml vitamin K and 7.5mM L-carnitine(

The decrease in the percentage of total deformities in the treatments with vitamin K or carnitine compared to the other treatments following dilution, cooling, and post-thaw. This might dut to the sperm buffalo membranes which is full with unsaturated free faty acid. The cell membarne with free faty acids is sustable to the damge causedd by frre radical formed during cyopreservation [15]. Vitamin K and amino acid like viatmin (carnitine) act as antioxidants. It is added to sperm that contain limited antioxidants, which cause high levels of ROS, oxidative stress, and DNA damage, which leads to a higher rate of abnormalities [26]. This effect is similar to the

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action of powerful antioxidants such as vitamins D, C, E, selenium, and zinc, and thus it works as an antioxidant that reduces the rate of abnoraml sperm[12,16,27]. Regarding the time factor, the reason for the decrease in the percentage of abnormalities following dilution compared to following cooling, and post-thaw can be related to the fact that cryopreserved semen causes the disarrangemnt of fats and proteins in the cell wall [28,29]. Thawing the semen following freezing causes a decrease in motility, viability, and increased in the abnormal sperm. This might happened due to the damage occure to the plasma membrane and changes in osmotic pressure as a result of recrystallization of water molecules inside the sperm, which lead to an increase in oxidative stress, causing an increase in reactive oxygen species and cell abnormalities [30.[

Conclusion

In conclusion, supplementation of semen extders with vitamin K or L-carnitine decreaseds the sperm abnormalties compared to control. Both vitamin K and L-carnitine maintain the intact sperm post-thaw comapred to other treatments. The current research brings pormising resultts regarding vitamin K and amino acid like vitamin (L-carnitine). More study is warrant to evaluate other sperm parameters on antioxidant and DNA fragmentation .

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