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Original Research

# Effect of vitamin B12 with the addition of extenders on some parameters of semen in Awassi rams

**Authors:**
**Ahmed Younis Saieed[1], Khalaf AH Al-Rishdy[2], Zainab A Mahadi[3] and Hobi A Abd-Alkareem[3]**

**Institution:**
1. General Directorate of Vocational Education, Ministry of Education, Iraq.

2. Department of Animal Production, College of Agriculture, University of Basrah, Iraq.

3. Department of Animal Production, College of Agriculture, University of Baghdad, Iraq.

**Corresponding author:**
**Hobi A Abd-Alkareem**

**ABSTRACT:**

This study was aimed to investigate the effects of vitamin B12 and different cryopreservation techniques on some Awassi ram sperm parameters and sperm mitochondrial apoptosis. Semen samples were collected form five Awassi rams, evaluated and pooled. Fresh semen was diluted with Tris extender containing 0, 0.5, 1, 2, 4µg/ml and was cooled at 5°C and equilibrated for 2h. The obtained Semen was packed in 0.25ml cryovial. The results of this study showed that there were significant ($P<0.05$) increase of nitrogen vapour for a period of 10 min in the study characteristics of semen in comparison with 5min of the samples exposed to nitrogen vapour. The addition of low concentrations of vitamin B12 enhances the studied characteristics in comparison with the optimal and high concentrations of vitamin B12 (0, 2, 4µg). There seen a significant effect of interaction between the exposed samples to nitrogen vapour for a period of 10 min and at 0.5 and 1µg concentration of B12 in the studied characteristics. Use of 0.5 and 1µg of vitamin B12 with nitrogen vapour technique (10min) improves the sperm parameters post- thawing.

**Keywords:**

Vitamin B12, Awassi rams, Frozen-thawed semen.

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## INTRODUCTION

The Reactive Oxygen Species (ROS) formed because of cooling, freezing and thawing ram semen (Watson, 2000) reduce spermatozoa motility (Matsuoka *et al*., 2012) and membrane function (Bucak *et al*., 2007) and increase mitochondrial apoptosis (Ahmed, 2016). There are a lot of Polyunsaturated Fatty Acids (PUFA) in the plasma membranes of spermatozoa, this is due to the role of cytoplasm that contains low concentrations of cleaning enzymes, and they are especially predisposed to the destruction induced by excessive ROS production (Halliwell, 1994). The group of vitamin B12 are considered as the water-soluble vitamins that functions as a coenzyme in a number of biochemical reactions, such as the metabolism of branched amino acids and methionine synthesis (Juanchi *et al*., 2000), because of its stability, also called the cyanocobalamin is the form that is typically used in vitamin enhancements.

The cyanocobalamin (vitamin B12) is active through cellular replication and DNA synthesis but maybe to save α-tocopherol and is previously being used as a treatment for male sterility in human beings (Eskenazi *et al*., 2005). Providing cyanocobalamin will lower the amount of ROS produced through the oxidative stress in human semen (Chen *et al*., 2001). There are several techniques used for freezing semen, including fast and slow freezing technology, which affects the vitality of semen after thawing (Ongun and Bucak, 2009) or the technique of exposing samples to nitrogen vapour prior to dipping in nitrogen liquid (Pfananani, 2012). Therefore, this study was conducted to know the effect of nitrogen vapour before dipping for 5 to 10 minutes and add vitamin B12 at various concentrations *viz*., 0, 0.5, 2, 4% to dilute the rams' semen and the interaction between them on the movement and integrity of the plasma membrane, dead sperm and apoptosis of mitochondria.

## MATERIALS AND METHODS

### Animals and semen collection

This study has been conducted at the College of Agriculture, University of Baghdad in cooperation with the High Institute of Infertility Diagnosis and Assisted Reproductive Technology, Al-Nahrain University. Five Awassi rams were exposed to the effect of different concentrations of vitamin B12 and different cryopreservation techniques on the post-thawed quality of ram semen cryopreserved in Tris.

Their body weight and age ranged between 3-2.5years and 80-75kg respectively. The Awassi ram semen was collected by artificial vagina designed for sheep and goats as well. The microscope tests of semen were processed before cryopreservation and cooling techniques.

### Semen processing

Semen were diluted with Tris solution containing vitamin B12 at different concentrations added at the rate of 0.5, 1, 2, 4µg and control, the dilution processed until reaching to 1:10 with 10% fresh egg yolk and glycerol 5%, semen was packed into 0.25ml cryovial (NUNC, Danmark). Diluted semen was then cooled slowly to 5°C, all concentrations would be left two hours to make equilibrium period. Nitrogen vapour technique was used in cryopreservation technique, the sample was exposed to it for 5min and 10min respectively and then immersed in liquid nitrogen. After one month, the sample would levered up and thawed in water bath at 37°C for 30s.

### Evaluation of frozen-thawed semen

The semen after thawing was evaluated by measuring the percentage of motility, Membrane Integrity (MI), dead sperm and mitochondrial apoptosis after one month from cryopreservation. Motility was assessed using a phase contract microscope (400x) with a heated stage (Chemineau *et al*., 1991).

The procedure described by Lomeo and Giambersio (1991) was used to determine the percentage of

**Table 1. Effect of different concentrations of vitamin B12 on the characteristics of Awassi ram sperm after thawing (mean± SEM)**

| S. No | Seminal characters (%) | 0µg | 0.5µg | 1µg | 2µg | 4µg |
|---|---|---|---|---|---|---|
| 1 | Motility | 33.70 ±0.36$^{c}$ | 38.900.61±$^{a}$ | 38.90 ±0.37$^{a}$ | 34.70±0.43$^{b}$ | 29.80±0.51$^{d}$ |
| 2 | Membrane integrity | 38.00 ±0.51$^{c}$ | 43.000.47 ±$^{a}$ | 42.20 ±0.41$^{a}$ | 39.50±0.38$^{b}$ | 34.40±0.40$^{d}$ |
| 3 | Dead sperm | 47.80 ±0.41$^{b}$ | 45.70 ±0.30$^{c}$ | 46.00 ±0.25$^{c}$ | 48.20±0.41$^{b}$ | 51.00±0.47$^{a}$ |
| 4 | Apoptosis Mitochondria | 75.00 ±0.36$^{b}$ | 69.20 ±0.71$^{e}$ | 70.20 ±0.41$^{d}$ | 73.70±0.36$^{c}$ | 76.40±0.33$^{a}$ |

Water Test (WT) in the positive sperm cells in each semen sample. The evaluation of 200 spermatozoa was done for evidence of swelling and curling change of the sperm tail. Dead cells separation was performed using eosin-nigrosin stain procedure described by Swanson and Beardon (1951). Disruption of the Mitochondrial Transmembrane Potential (MTP), which is one of the earliest intracellular events that occur following the induction of apoptosis, was detected using apoptosis detection, mitochondria bioassay™ kit (US Biological). In healthy cells, the dye accumulates and aggregates in the mitochondria, showing a bright red fluorescence (λem = 488-590 nm), while in apoptotic cells with altered MTP, the dye in its monomeric form cannot show off in the mitochondria and stays in the cytoplasm with fluorescent green (λem = 488-530 nm) (Ying-Chen *et al.*, 2001).

**Statistical analysis**

The data were statistically analyzed using SAS (SAS, 2012). Sperm parameters post-cryopreservation using different concentrations of vitamin B12 and nitrogen vapour technique were analyzed using Complete Randomized Design (CRD). Duncan mean test (Duncan, 1955) was used for the treatment mean comparisons.

**RESULTS**

The results of statistical analysis recorded high percentage of individual sperm motility with concentrated (0.5, 1µg) vitamin B12 (38.90±0.61, 38.90±0.37) respectively. Whereas the highest percentage of membrane integrate was recorded for 0.5µg concentration of vitamin B12 (43.00±0.47%). The percentages of apoptosis in mitochondrial and dead sperms were recorded in 4µg concentration with the value of 51.00±0.47, 76.40±0.33% respectively (Table 1).

The results showed that when samples were exposed to a period of nitrogen vapour for 5 to 10 minutes, high significant difference was noted between the staged traits. Table 2 illustrated that in the period of 10min sperm motility and membrane integrity recorded 36.00±0.72, 40.40±0.66% respectively, while the period of 5min recorded the highest percentage of dead sperm and mitochondrial apoptosis 48.52±0.48,74.00±0.51% respectively.

No significant differences were seen between 0.5 and 1µg concentrations in the period of 10 min for exposing nitrogen vapour for analyzing properties of sperm motility after thawing, lowest percentage of motility sperm (28.6%) in the period of 5min Figure 1.

The interaction between vitamin B12 concentrations and nitrogen vapour exposing periods (5,10min) illustrated decreasing membrane integrity in (4µg)

**Table 2. Effect of exposing samples to nitrogen vapour technique for the period of 5 and 10 minutes (mean±SEM)**

| S. No | Seminal characters | 5min | 10min |
|---|---|---|---|
| 1 | Motility (%) | 34.16±0.71$^{b}$ | 36.08±0.72$^{a}$ |
| 2 | Membrane integrity (%) | 38.44 ±0.69$^{b}$ | 40.40±0.66$^{a}$ |
| 3 | Dead sperm (%) | 0.48±48.52 $^{a}$ | 46.96 ±0.35$^{b}$ |
| 4 | Apoptosis Mitochondria (%) | 0.51±74.00 $^{a}$ | 71.80 ±0.65$^{b}$ |

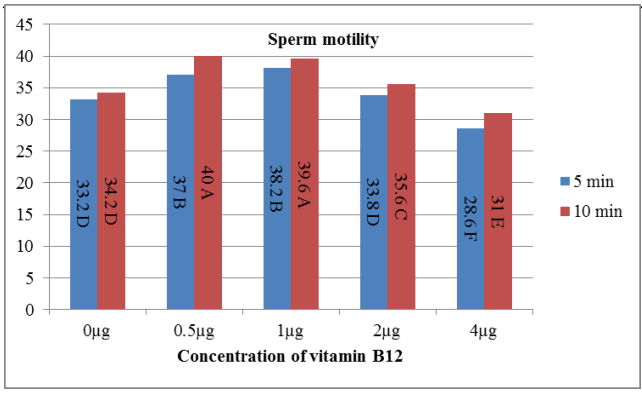


**Figure 1. Means of sperm motility after freezing thawing process using extenders with different concentrations of vitamin B12**

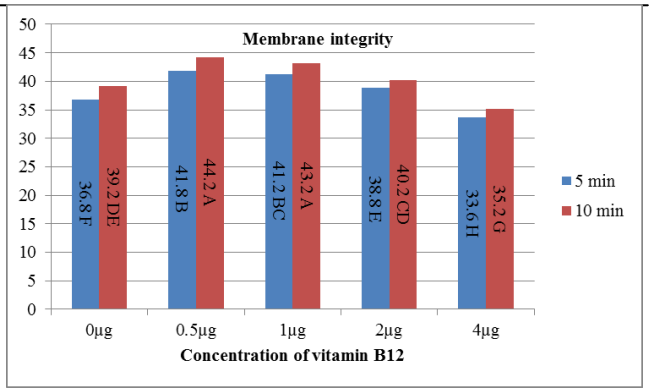


**Figure 2. Means of membrane integrity after freezing thawing process using extenders with different concentrations of vitamin B12**

concentration of vitamin B12 and control treatment in comparison with other treatments (Figure 2). The results showed that 0.5µg concentration of vitamin B12 recorded the lowest percentage of dead sperms in the period of 10min (45.20%), while the concentration 4µg of vitamin B12 recorded the highest percentage (52.20%) in the period of 5min in comparison with the other treatments (Figure 3). The concentrations 0µg/5min and 4µg/5min of vitamin B12 recorded highest percentage of apoptosis in mitochondria (75.8, 77.2%) respectively, while concentration 0.5µg/10min of vitamin B12 showed lowest percentage of apoptosis in mitochondria (67.2%) (Figure 4).

## DISCUSSION

The deterioration of semen quality is often due to the cold shock, freezing and thawing which affect the metabolic capacity of sperm due to the release of enzymes and ions from the sperm head (Curry, 2000; Morris *et al.*, 2012). The stages of the freezing process lead to mechanical stress of the plasma membrane of the sperm and the loss of osmotic system, resulting in a change in the amount of water inside the sperm (Hammerstedt *et al.*, 1990). In the current study, the use of low concentrations of vitamin B12 improved semen characteristics compared with the control groups with an addition of 2 and 4µg. Addition of vitamin B12 to the semen extender could improve the sperm motility of ram and bull during cryopreservation which is consistent with the coenzyme A activity of vitamin B12 (Cai *et al.*, 2004). While Ha and Zhao (2003) mentioned that adding vitamin B complex to diluted semen of the ram improved the quality of semen during cryopreservation. The results of our study showed that there were

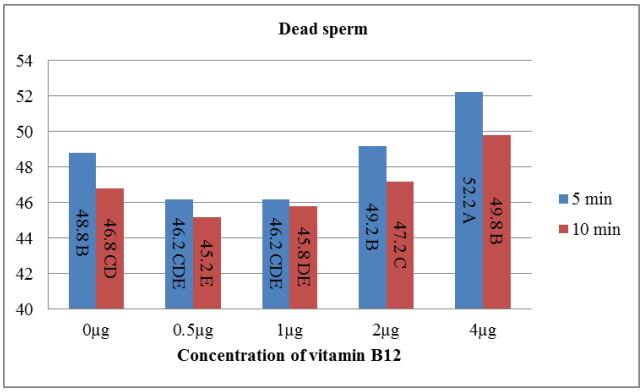


**Figure 3. Means of dead sperm after freezing thawing process using extenders with different concentrations of vitamin B12**

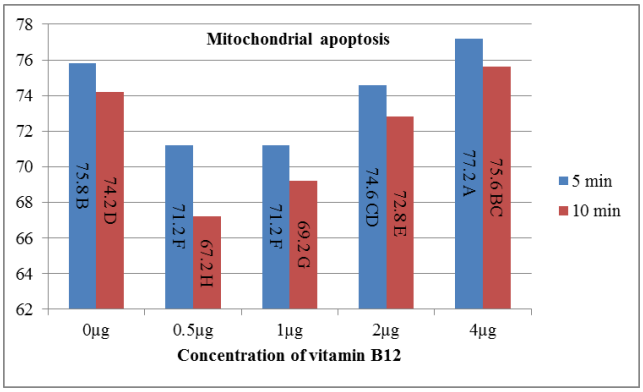


**Figure 4. Means of mitochondrial apoptosis after freezing thawing process using extenders with different concentrations of vitamin B12**

significant ($p<0.05$) increase in the characteristics of semen which are that exposed to nitrogen vapour technique for 10min in comparison with that exposed to the nitrogen vapour for 5min. The study by Pfananani (2012) for comparison of slow freeze technique and the technique of exposing the samples to nitrogen vapour for 10 minutes before dipping with liquid nitrogen. The ram sperm indicated a significant superiority at the slow freezing technique. It is important to note that the process of freezing and thawing limits the activity of the antioxidants, making the sperm more susceptible to free radicals and accelerating the release of programmed apoptosis and fragmentation to DNA (Lasso *et al*., 1994; Wang *et al*., 2003). In the study of Nur *et al*. (2011), the effect of the slow and rapid freezing technique on the sperm was significantly higher ($p<0.05$) for the sperm motility after the thawing between the two methods (42.8±8.8 and 36.5±9.9) respectively, for the membrane integrity, fragmentation of DNA and mitochondrial apoptosis between the two methods. In this study, the interaction between the concentration of 0. 5 and 1 µg of vitamin B12 and the exposure of the samples to the nitrogen vapour of 10 minutes resulted in the improvement of semen characteristics after thawing. Chen *et al*. (2001) observed a positive correlation between vitamin B12 concentration in the seminal plasma and sperm concentration where vitamin B12 showed a reduction in ROS due to the oxidative stress. Ha and Zhao (2003) showed that the decrease of the proportion of Glutamic Oxaloacetic Transaminase (GOT) in the seminal plasma of rams during the addition of vitamin B12 was seen in the diluted and this is an important indicator of the improvement of motility of sperm during activity cooling. The reason for the addition of vitamin B12 is to increase the effectiveness of the antioxidant enzymes Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx) which reduced the oxidative stress and improve characteristics of the rams after thawing (Asadpour *et al*., 2012). The results of our study on the role of vitamin B12 in improving the motility and viability of the bulls' sperm (Cai *et al*., 2004) and pigs' sperm (Mello *et al*., 2013).The high concentrations of vitamin B12 have a toxic effect on the sperm of bulls, however, there is no explanation for the deterioration in sperm characteristics (Hu *et al*., 2011).

## CONCLUSION

From the results of the present study, it is noted that nitrogen vapour technique is a simple technique and easy to perform. Use of 0.5 and 1µg of vitamin B12 with nitrogen vapour technique (10min) improves the sperm parameters post- thawing.

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