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*Original Article*

# Effect of Vitamin D3 on some Antioxidant Parameters in Chilled Semen in Awassi Ram

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**Abstract**

To sustain the viability of the sperm of farm animals, the sperm is chilled. However, reactive oxygen species (ROS) may damage it, resulting in oxidative stress and decreased sperm viability. This study aimed to assess the various concentrations of vitamin D3 as antioxidants in the chilled sperm of Awassi. This study was performed on 23 ejaculates from three Awassi rams. The samples were combined, diluted with Tris-egg yolk extender (1:10), and then divided into aliquots. Aliquots were treated with three vitamin D3 concentrations (T1=0.02, T2=0.004, and T3=0.002 g/ml) and one control without the addition of vitamin D3. The experimental and control groups were chilled to reach 5 ºC. Following treatment, the samples were centrifuged at 2,000 RPM for 20 min at 0 and 72 h after the treatment. Until evaluation, the seminal plasm was stored in a freezer at 20 ºC. In this study, the antioxidant activity of vitamin D3 was evaluated using malondialdehyde (MDA), ROS, total antioxidant capacity (TAC), superoxide dismutase (SOD), and catalase (CAT). The SAS software was used to analyze variance on repeated measures with a single factor. The results indicated that the TAC and SOD were considerably higher in T1, compared to that in T0, T1, and T2. In addition, CAT was considerably higher in T2 than in T0, T1, and T3. However, ROS and MDA did not differ significantly among the experimental groups. Despite the absence of a statistically significant difference among experimental groups, MDA decreased quantitatively on T1, relative to other experimental groups. In conclusion, a deficiency in vitamin D3 has a potential antioxidant capability, introducing a novel method for extending sperm storage.

**Keywords:** Chilled semen, Awassi ram, Vitamin D3, Antioxidant

## 1. Introduction

Assisted reproductive techniques in sheep have not been used widely, compared to other domestic animals (1). The chilled semen method following dilution has been used in farm animals to maintain semen viability (2). However, semen viability might be damaged with prolonged semen storage, resulting in reduced fertility (3-5). This reduction in sperm quality is generally due to the accumulation of reactive oxygen species (ROS) in seminal plasma caused by sperm metabolism (6), produced by lipid peroxidation. This byproduct is initiated by the intra and intercellular reaction activity of enzymes, which leads to the formation of free radicals (7).

Free radicals lead to a typical phenomenon called oxidative stress (8). This phenomenon happens due to changes in the balance between antioxidants and pro-oxidants. Pro-oxidant production increment leads to a change in the homeostasis of the cells and decreases cell survival (9). It damages the cell membrane and genes (10). In addition, the ram sperm membrane is rich in polyunsaturated free fatty acid, which is more likely to face oxidative stress and then lipid peroxidation, resulting in irreparable cell membrane damage, DNA fragmentation, and loss of motility (11, 12), which thereby decreases fertilization ability. Therefore, it is vital to supplement antioxidant

materials to the chilled semen of sheep to prevent oxidative stress (13).

Antioxidant materials, such as vitamins, must be used to reduce oxidative stress and ROS accumulation in the cooled semen. According to previous studies, vitamin D3 is one of the sources of antioxidants (14). Results of our recent research on sheep semen dilution (15) have indicated that the addition of different concentrations of vitamin D3 to chilled semen prolongs the sperm individual motility for up to 72 h. In the present research, the treatment might reduce the effect of ROS accumulation, which helps prevent lipid peroxidation and maintain sperm motility (16).

Furthermore, supplementation of frozen semen extender with different vitamin D concentrations in bull enhanced the sperm parameter in the freezing thaw procedure (17). In the aforementioned study, a freezing semen extender that was supplemented with a high dose of vitamin D (50 ng/mL) maintained sperm integrity, viability, and motility, compared to the control group. It was concluded that vitamin D has a promising protective antioxidant activity on semen extenders (17). In humans, supplementation of vitamin D for infertile men with vitamin D deficiency has a positive effect on semen and antioxidant parameter (18). In the above-mentioned study, supplementation of vitamin D (4000 IU for 12 weeks) for infertile men (sperm motility≤40% and vitamin D level≤30 ng/mL) enhanced their sperm parameters and increased their antioxidant parameters.

The present research is continuing previous published research to study the effect of vitamin D3 on the chilled semen of Awassi. Accordingly, the current study aimed to evaluate the effects of different concentrations of vitamin D3 as antioxidants on the chilled semen of Awassi. It was hypothesized that supplementation of the semen of Awassi with a low concentration of vitamin D3 might enhance the antioxidant parameters.

## 2. Materials and Methods

The current study was conducted on three mature Awassi rams (average weight: 60 kg and 2-5 years old). An artificial vagina (made in our laboratory) was used to collect semen samples. To prevent individual differences, 23 collected ejaculates were pooled and then extended with a Tris-egg yolk extender (1:10) at 37 ºC. Samples were divided into experimental and control groups. The experimental groups received three vitamin D3 concentrations (T1=0.02, T2=0.004, and T3=0.002 gm/ml), while the control group received no supplement. Experimental and control groups were cooled to 5 ºC. Samples were centrifuged at 1,200 RPM for 15 min at 0 and 72 h after the treatment. The seminal plasma was kept in a freezer at 20 ºC until evaluation.

Malondialdehyde (MDA) is an indicator used for the evaluation of sperm membrane damage produced by ROS. The MDA concentration was evaluated to determine the interaction between thiobarbituric acid and MDA (19). Briefly, 0.4 mL seminal plasma was added to 0.6 TCA-TBA-HCl reagents mixed well, and then boiled for 10 min. After cooling, 1 mL of fresh NaOH (1N) was added. In the blank solution, the procedure was the same as that of the MDA, except that distal water was used instead of seminal plasma. Both the blank and sample absorbance were read at 535 nm wavelength.

### 2.1. Reactive Oxygen Species

The ROS is an indicator for the evaluation of the sperm for oxidative stress, estimated by the FOX2 method (20). It is based on the ferrous ion/oxidation complex. Briefly, 140 µl of the sample was added to 900 µl of reagent 1 (150 µM xylenol orange, 140 mM NaCl, and 1.35 M glycerol in 25 mM $H_2SO_4$) and 44 µl of reagent 2 (5 mM ferrous ammonium sulfate and 10 mM o-dianisidine dihydrochloride in 25 mM $H_2SO_4$). Samples were vortexed and then incubated at room temperature for 30 min. Following incubation, the absorbance of the samples was measured by spectrophotometer (V-550 UV-vis) at a wavelength of 650 nm.

### 2.2. Total Antioxidant Capacity

Total antioxidant capacity (TAC) was determined according to the method described by Apak, Guclu

(21). Accordingly, 50 µl of seminal plasm, working standard solution, and distal water was added to 1 mL of Copper (II) chloride solution in each tube (sample, stander, and blank), respectively. Subsequently, it was vortexed very well, then 1 mL Neocuproine solution and 1 mL Ammonium acetate (NH4Ac) buffer were added to each tube, and incubated for 30 min at 37 ºC. Following incubation, absorbance was measured by spectrophotometer at 450 nm wavelength.

### 2.3. Superoxide Dismutase

Superoxide dismutase (SOD) activity was determined according to the method described by Marklund and Marklund (22). The principle of this method depends on the competition between the pyrogallol autoxidation by $O_2\bullet^-$ and the dismutation of this radical by SOD. Briefly, fifty µl from seminal plasm and distal water were added to 1 mL Tris buffer for each tube (sample and control). Wavelength was read at 420 nm against Tris-EDTA at 0 and 1 min after the addition of 1 mL of pyrogallol.

### 2.4. Catalase

Catalase (CAT) activity was determined according to the method described by Goth (23). Briefly, 0.2 µl of seminal plasm and 1 mL of substrate buffer were incubated at room temperature for 1 min. The enzyme activity was suspended by the addition of 1 mL of 32.4 mM ammonium molybdate. The wavelength of the molybdate with hydrogen peroxide complex was measured by a spectrophotometer at 405 nm.

### 2.5. Statistical Analysis

One-way analysis of variance and repeated measures analysis were used in the SAS software (24) to study the effect of different concentrations of vitamin D3 on some antioxidant parameters of the chilled semen of Awassi. Duncan's multiple range test was also used to compare the differences between experimental groups (25).

## 3. Results

In the current research, MDA was significantly lower in T3, 72 h after the treatment ($P \leq 0.01$; 16.63±0.41), compared to T0, T1, and T2 (24.35±1.01, 23.36±1.95, and 27.26±3.44, respectively) (Figure 1). It is essential to mention that the MDA level increased in all experimental groups at 72 h after the cooling at 5 ºC, except for T3, which decreased in the same parameter. However, ROS did not undergo any significant changes within and among groups (Figure 2).

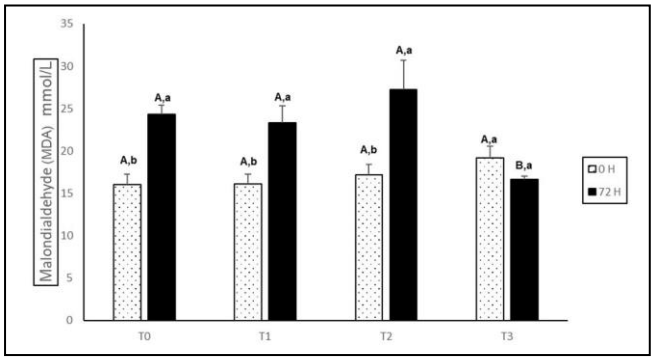


**Figure 1.** Effect of adding different vitamin D3 concentrations as antioxidants on (mean ± SEM) malondialdehyde level on local chilled Awassi ram semen. Semen samples were evaluated at times 0 hours, and 72 h following treatments (T0= control (no treatment), T1=0.02, T2=0.004, and T3=0.002 gm/mL vitamin D3). $^{A \text{ and } B}$ letters among groups differ significantly ($P \leq 0.05$). $^{A,b}$ letters within each treatment differ significantly ($P \leq 0.05$)

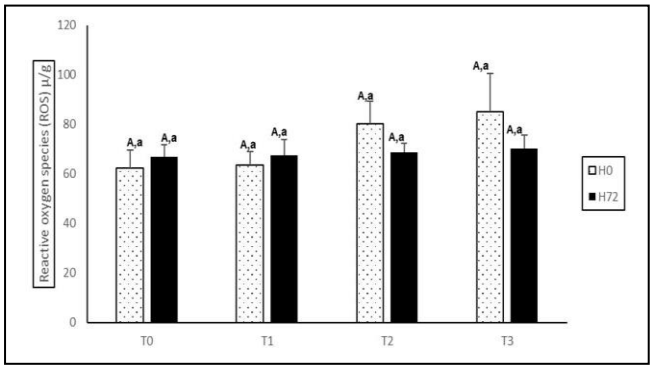


**Figure 2.** Effect of adding different vitamin D3 concentrations as antioxidants on (mean ± SEM) Reactive oxygen species (ROS) on local chilled Awassi ram semen. Semen samples were evaluated at times 0 hours, and 72 h following treatments (T0= control (no treatment), T1=0.02, T2=0.004, and T3=0.002 gm/mL vitamin D3). $^{A, a}$ letter with the same letters among and within treatment were not significantly different

The TAC was significantly greater in T3 at 72 h ($P \leq 0.0001$; 3117.97± 175.6) following the treatment, compared to T0, T1, and T2 (2218.42± 32.64, 2505.40

±108, and 2327.97± 65.38.6, respectively) (Figure 3). In addition, the TAC parameter decreased significantly in the T0, T1, and T2 groups over time, but increased in the T3 group.

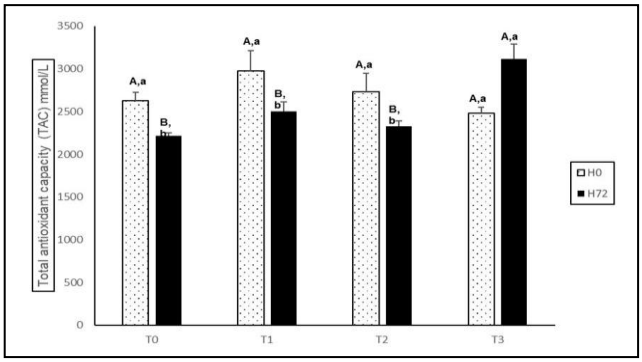


**Figure 3.** Effect of adding different vitamin D3 concentrations as antioxidants on (mean ± SEM) total antioxidant capacity on local chilled Awassi ram semen. Semen samples were evaluated at times 0 hours, and 72 h following treatments (T0= control (no treatment), T1=0.02, T2=0.004, and T3=0.002 gm/mL vitamin D3). [A and B] letters among groups differ significantly (P<0.05). [a,b] letters within treatment differ significantly (P<0.05)

The SOD was significantly greater on T3 at 72 h (P<0.001; 90.11±2.83) following the treatment, compared to T0, T1, and T2 (72.37±5.18, 70.50±1.48, and 70.73±5.18, respectively) (Figure 4). However, the SOD parameter in T1 decreased significantly at 72 h, compared to time 0 h after the treatment.

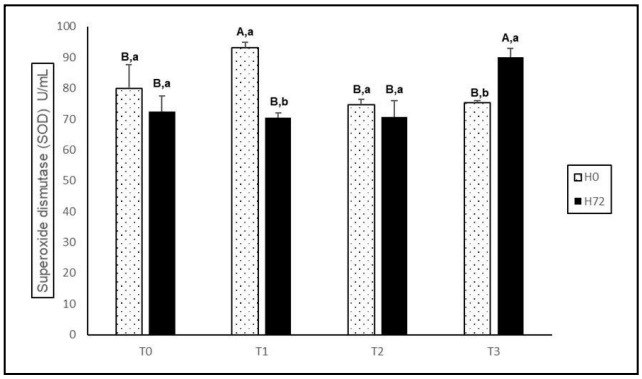


**Figure 4.** Effect of adding different vitamin D3 concentrations as antioxidants on (mean ± SEM) superoxide dismutase (DOS) on local chilled Awassi ram semen. Semen samples were evaluated at times 0 hours, and 72 h following treatments (T0= control (no treatment), T1=0.02, T2=0.004, and T3=0.002 gm/mL vitamin D3). [A and B] letters among groups differ significantly (P<0.05). [a,b] letters within treatment differ significantly (P<0.05)

The CAT level was significantly greater on T2 at 72 h (P<0.01; 52.24 ±2.09), following treatment, compared to T0, T1, and T3 (45.56± 2.30, 45.04 ± 1.64, and 44.59 ± 0.43), respectively (Figure 5). It is important to mention that the CAT increased significantly over time on T0, T1, and T3 (Figure 5). However, the CAT parameter in T2 did not change over time.

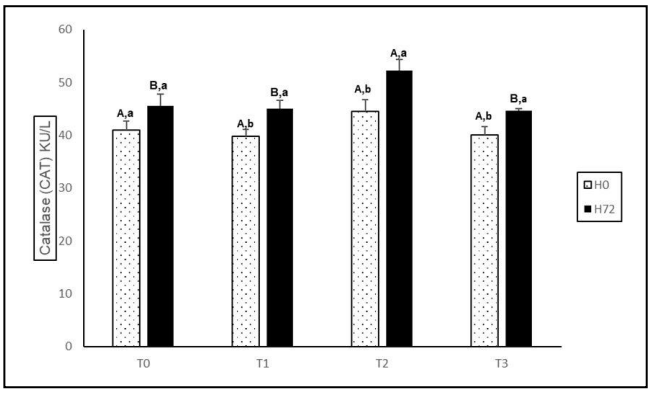


**Figure 5.** Effect of adding different vitamin D3 concentrations as antioxidants on (mean ± SEM) catalase (CAT) on local chilled Awassi ram semen. Semen samples were evaluated at times 0 hours, and 72 h following treatments (T0= control (no treatment), T1=0.02, T2=0.004, and T3=0.002 gm/mL vitamin D3). [A and B] letters among groups differ significantly (P<0.05). [a,b] letters within treatment differ significantly (P<0.05)

## 4. Discussion

This research continued the previously published research to test the effect of vitamin D3 as an antioxidant on the chilled semen of Awassi. Based on our knowledge, this is the first study to find the effect of vitamin D3 on the cooled semen of Awassi ram.

The results showed that the MDA was significantly lower in T3 at 72 h after the treatment (P≤0.01; 16.63 ± 0.41), compared to T0, T1, and T2. This result was supported by those of the studies performed by Khalifa (26), (27), who concluded that MDA activity decreased with the addition of antioxidants. Norouzabad et al. concluded that oral supplementation of vitamin D for infertile men changed the MDA seminal plasma parameter (18). The MDA produces in the sperm membrane by lipid peroxidation (28). An increase in MDA is a marker of high lipid peroxidation, which negatively correlates with sperm motility and infertility

(29, 30). This result is consistent with that of the present study which indicated that the addition of vitamin D3 to the semen extender enhanced semen parameters, compared to the control group (16). However, ROS was not significantly different within and among groups. This result was not supported by the study performed by Wu, Dai (27), who reported that ROS activity decreased with antioxidants.

The TAC was significantly greater in T3 at 72 h ($P \leq 0.0001$; 3117.97±175.6) following treatment, compared to T0, T1, and T2 (2218.42±32.64, 2505.40±108, and 2327.97±65.38.6, respectively). This result was supported by Maghsoumi-Norouzabad, Zare Javid (18), who reported that treatment of infertile men with different concentrations of vitamin D enhanced the TAC index, compared to untreated men. Moreover, the addition of vitamin D to the semen extender of bulls enhanced the semen parameter following cryopreservation (17). The TAC is an indicator of antioxidants for semen samples (13), which is consistent with the findings of this research.

The SOD and CAT are enzymatic antioxidants that help reduce free radicals and oxidative stress (13). The CAT enzyme is located in peroxisomes which convert hydrogen peroxidase to water and $O_2$ (31). In sheep, liquid storage semen supplementation with CAT reduced the detrimental effect of prolonged cooled semen storage (32). In humans, low sperm motility is associated with a low level of CAT, compared to normal sperm motility (33). This provides essential evidence that the level of CAT plays a potential role in seminal plasma following liquid storage (34, 35).

It should be mentioned that SOD also converts hydrogen peroxidase to water and $O_2$ (31). It catalyzes and scavenges the superoxide anion. The SOD is found in seminal plasma (33) and used as a marker of reduction in lipid peroxidation (36), DNA fragmentation (33), oxidative stress (35), and positive correlation with sperm motility (15, 27). In addition, SOD is induced by vitamin D3 in mice (37). Therefore, based on the above-mentioned evidence, vitamin D might play a vital role in sperm protection.

In conclusion, a low dose of vitamin D3 supplementation to the Awassi semen extender increased antioxidant parameters, such as TAC and SOD. At the same time, vitamin D3 decreases the MDA level. Based on our knowledge, this study was the first to report the effects of vitamin D, as an antioxidant, on chilled semen extenders.

## Authors' Contribution

Study concept and design: M. H. A. B. and H. M. H. H.
Acquisition of data: M. H. A. B.
Analysis and interpretation of data: M. H. A. B. and H. M. H. H.
Drafting of the manuscript: M. H. A. B. and H. M. H. H.
Critical revision of the manuscript for important intellectual content: H. M. H. H.
Statistical analysis: H. M. H. H.
Administrative, technical, and material support: M. H. A. B.
Study supervision: H. M. H. H.

## Ethics

The Ethical protocols of the study were approved by the Department of Animal Production Council, Al-Qasim Green University, Babylon, Iraq.

## Conflict of Interest

The authors declare that they have no conflict of interest.

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