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Effects of vitamin B12 addition on hydrogen peroxide generation and motility characteristics of fresh and chilled ram spermatozoa

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ABSTRACT

The antioxidant ability of vitamin B12 (VitB12) and its effects on motility characteristics of fresh and chilled ram spermatozoa were investigated. Three concentrations of VitB12 including 1, 2 and 4 mg/ml were added to tris media and spermatozoa samples collected from five Awassi rams were incubated in these media at 37 and 5 °C for 2 and 24 hours respectively. Formation of hydrogen peroxide (H_2O_2) was estimated using a fluorometric assay and motility characteristics were analyzed using computer aided sperm analyzer (CASA). A significant decrease ($P<0.05$) of H_2O_2 formation was noted after VitB12 addition and the generation levels decreased when the concentration of VitB12 was increased. The values of motility characteristics including percentage of motility (MOT %), percentage of sperm showing progressive motility (PMOT %) and average path velocity (VAP) were increased ($P<0.05$) after 2 hours of incubation with 1 and 2 mg/ml of VitB12 for the fresh spermatozoa and after 24 hours of incubation for the chilled ones. In contrast, 4 mg/ml of VitB12 was able to decrease ($P<0.05$) the motility values for the fresh and chilled spermatozoa types. No differences were noted for percentage straightness (STR %) as well as percentage linearity (LIN %). It was concluded that VitB12 has an antioxidant capacity expressed by its ability to suppress H_2O_2 formation. The effects of this compound were related to the used concentration. VitB12 can be used as an antioxidant agent and motility stimulant in the semen media used for ram spermatozoa.

Keywords: vitamin B12, spermatozoa, reactive oxygen species, antioxidant

INTRODUCTION

It is well known that vitamins are organic compounds which play an important role in the growth and the development of different cell types. Moreover, the classification of vitamins largely depends on their chemical nature and their specific function (Sinbad et al., 2019). One of the most important vitamins is B12 (VitB12) also known as cobalamin. This water-soluble vitamin found in foods of animal origin was essential in nutrition with a complex structure containing cobalt (Green et al., 2017). In humans, a clear correlation between VitB12 and male reproductive performance has been demonstrated and it was suggested as one of the candidate drugs to manage male infertility (Chatterjee et al., 2006). This vitamin was essential for spermatogenesis and its supplementation enhanced rats epididymal sperm concentration and reinforced spermatogenic recovery (Chen et al., 2001; Beltrame and Saeso-Cerri, 2017). Furthermore, Ha and Zhao (2003) indicated that B complex vitamins improve post-thaw motility and protect the membrane integrity of ram spermatozoa during cryopreservation.

On the other hand, reactive oxygen species (ROS) including agents such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radical (HO·) and hydrogen peroxide (H_2O_2) could provoke chemical alteration which results in the injury of the cell's role (Vassalleet et al., 2008). The generation of ROS from the spermatozoa was increased in the presence of cryo-

damaged, dead, or morphologically abnormal sperms (Ball et al., 2001; Alomar, 2018). Hydrogen peroxide, which is the most important member of the ROS family, was the major ROS agent responsible for the loss of motility that occurred in stress response in spermatozoa (Alomar et al., 2018a). However, the members of ROS family can be neutralized by different antioxidant systems including reduced glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT), superoxide dismutase (SOD) as well as vitamins C, D, and E which also serve as a defence mechanism against lipid peroxidation (Agarwal, and Prabakaran, 2005; Bansal and Bilaspuri, 2011). In addition to the previous antioxidants systems, vitamin B12 may play a key role in the oxidative stress cycle (Sinbad et al., 2019). Indeed, accumulated evidence from human and animal investigations suggests that lower VitB12 status was related to higher oxidative stress (Banihani, 2017; Van de Lagemaat et al., 2019). Moreover, clear evidence from *in-vitro* studies showed that VitB12 may possess antioxidant property (Moreira et al., 2011). In this regard, Chen et al. (2017) suggested that VitB12 possess a direct super-oxide antioxidant mechanism.

The use of fresh and chilled ram spermatozoa types in the different assisted reproductive technologies seem to be an important option as the use of cryopreserved ones. In fact, it is technically easier to collect fresh samples and to chill than to freeze semen. Nevertheless, fresh or chilled semen is less expensive than frozen semen. However, to achieve the most efficacious use of ram semen, antioxidants supplementation is an important choice to improve semen preservation conditions. Indeed, the use of antioxidants *in vitro* could be useful to optimize certain desired semen parameters. Moreover, an understanding of the generation and removal of ROS members especially H_2O_2 along with their effects on normal sperm physiology as motility status could help and develop ram semen preservation methods. For that, this study aimed to analyze the antioxidants' ability of VitB12 and its effects on motility characteristics of fresh and chilled ram semen.

MATERIALS AND METHODS

Site description, animals, semen collection and ethical approval

This study was carried out at Der-Al-Hajar Animal Production Research Station, 33 km south-east of Damascus. Semen was obtained in June from five sexually experienced Awassi rams, aged between 3 and 4 years. Semen samples were collected with the aid of an electro-ejaculator (Minitube - Electro Ejaculator, Tiefenbach, Germany) administrating a series of cycles pulses of short electrical stimuli with each cycle (two seconds impulse, then two seconds interval) delivering a slightly higher intensity (from 0 Volt to 20 Volt maximum) to obtain semen. Upon collection, the semen was evaluated for its general appearance and volume. Sperm concentrations were calculated using a hemocytometer (cell counting chamber; Neubauer Improved Marienfeld, Germany). An initial analysis of sperm motility was performed using CASA system (Hamilton Thorne Biosciences, Version 12 CEROS, Beverly, USA). Sperm samples with a motility score ≥ 75% of motile sperm and a concentration of ≥ 1 x 10^9 spermatozoa/ml were employed. All ejaculations with no or poor motility status or less than 1 x 10^9 spermatozoa/ml were immediately excluded before conducting the analyses. It must be noted that the present study was approved by the Local Scientific and Ethical Committee of the Atomic Energy Commission of Syria (AECS), Damascus, Syria (permit number36/ZM1-2020).

Chemical, media preparation and experimental design

All chemicals were purchased from Roth (Carl Roth Gmbh-Karlsruhe-Germany). Two tris media were employed in the present study, the first is tris based medium for the fresh semen samples and the second is tris – egg yolk medium for the chilled semen samples. Tris based medium was prepared as a 300 mOsm/kg solution contained the following: 2.44 g tris (hydroxymethyl) aminomethane, 1.36 g citric acid monohydrate and 1 g glucose in 100 ml of distilled water and this medium was held constant at pH 7. The tris –egg yolk medium was

prepared from 80 ml of the based medium and 20 ml of egg yolk was added to complete the final volume of this medium.

Four experiments were conducted in the present work using 30 ram ejaculates. To diminish the effect of individual variation between the rams a mixture of semen from the five animals was used in each assay. In the first and second experiments, the level of hydrogen peroxide formation from fresh and chilled spermatozoa with 1, 2 and 4 mg/ml of vitamin B12 and without (control) was determined. In the first experiment fresh spermatozoa at 10 x 10^6 spemtozoa/well were incubated for two hours in tris based medium at 37 °C and the hydrogen peroxide level was determined during 120 min of incubation in the fluorometer system. In the second experiment chilled spermatozoa at 10 x 10^6 spemtozoa/well were incubated for 24 hours at 5 °C in the refrigerator and H_2O_2 level was then determined using the fluorometer system. These two experiments were replicated for three times.

In the third and fourth experiments motility characteristics of fresh and chilled at 25 x 10^6 spemtozoa/ml incubated in tris based medium and tris egg yolk medium with1, 2 and 4 mg/ml of VitB12 and without (control) were analyzed after two hours of incubation at 37 °C in water bath for the fresh samples and after 24 hours of incubation at 5 °C in the refrigerator for the chilled ones. Each of the third and the fourth experiment was replicated for three times.

Measurement of hydrogen peroxide

Amplex red (10-acetyl-3, 7-dihydroxyohenoxazine, Molecular Probes) was used to monitor hydrogen peroxide (H_2O_2) generation. In the presence of horseradish peroxidase, amplex red reacts with H_2O_2 in a 1:1 stoichiometric reaction to produce resorufin, a highly fluorescent end product. A stock of amplex red was prepared in DMSO (10mM), while horseradish peroxidase was prepared in phosphate buffer (450 unit/ml, pH 7.5). Both stocks were stored at - 20 °C until the assay. Twenty µl of the sperm suspensions containing 10 x 10^6 of the fresh and chilled spermatozoa types were added to 96-well plates (Nunc, Roskilde, Denmark). Hydrogen peroxide standards (100, 50, 25, 12.5, 6.25 and 3.12µM) were prepared extemporaneously in tris based medium for the fresh samples and in tris egg-yolk medium for the chilled spermatozoa. Eighty µl of the medium containing 40 µM amplex red and 1 unit of horseradish peroxidase/ml were added to each well. The microplates were incubated at 37 °C for 120 min for the fresh spermatozoa in the fluorometer system (Fluoroskan Ascent FL; Thermo Fisher Scientific; Vantaa; Finland) and the fluorescence was recorded at intervals for 30, 60 and 120 min. On the other hand, as our fluorometer system could not refrigerate the samples, the chilled spermatozoa samples were measured directly for one time only after chilling the spermatozoa at 5 °C for 24 hours in the refrigerator. The generation of H_2O_2 was measured using an excitation wavelength, 530 nm and an emission wavelength, 590 nm. Sperm samples, standard solutions and blanks were assayed in duplicate. The concentration of H_2O_2 was determined from the standard curve based on the blank-corrected fluorescence for each measured time point and expressed in µM.

Motility analyses

The motility characteristics of the sperm were assessed by computer aided sperm analyzer (CASA), using the Hamilton-Thorne motility analyzer (HTM). Five microliters' aliquots of diluted spermatozoa were placed in the system lame (2X-CEL of dual sided sperm analysis chamber of 20 µm depth for the Hamilton Thorne Bioscines System) and loaded into the analyzer. At least three fields were counted for each sample (each field counted about 150-200 spermatozoa). The motility characteristics included in the analysis were: percent motility (MOT %), average path velocity (VAP, µm/s), percent linearity (LIN %), percent straightness (STR %), and the percent of sperm showing progressive motility (PMOT %; VAP ≥ 75 µm/s and straightness STR ≥ 80%). The HTM system settings of ram spermatozoa are presented in Table (1).

Table 1. The settings for the Hamilton Thorne Biosciences system version 12.3 used to evaluate ram semen

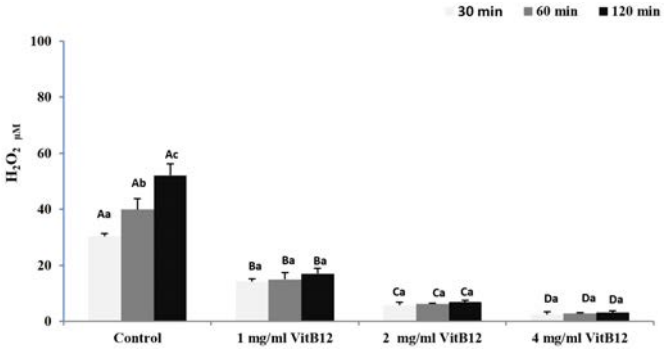
Parameter	Settings
Frame rate (Hz)	60
Frames acquired (no)	30
Minimum contrast	60
Minimum cell size (pixels)	5
Low average path velocity (VAP) cut off	21.99
Low straight-line velocity (VSL) cut off	6
Non-motile head size (pixels)	5
Non-motile head intensity	55
Static size limit (min/max)	0.60/8
Static intensity limit (min/max)	0.20/1.50

Statistical analysis

Statistical analysis was conducted with the Minitab program (Minitab Coventry, United Kingdom). The normality of values distribution was first tested with the Shapiro-Wilk test. Data were subjected to a factorial analysis of variance (ANOVA, general linear model procedure, GLM) followed by multiple pairwise comparisons using a post-hoc (Tukey test). The threshold of signification was set at $P<0.05$.

RESULTS

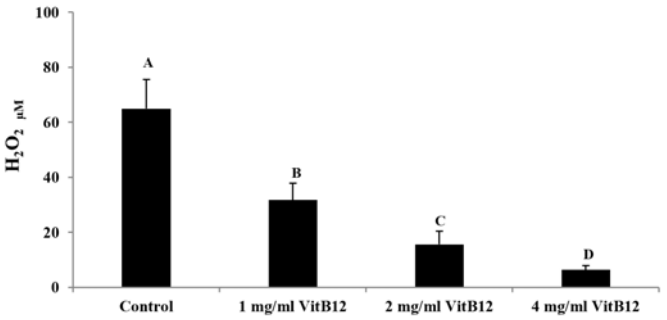
Figure 1 shows hydrogen peroxide generation from fresh ram spermatozoa with and without B12 addition during 120 minutes of incubation in tris based medium. Significant differences ($P<0.05$) between the three time points (30, 60 and 120 min) were observed for the control group. When the VitB12 was added, the production of H_2O_2 at each B12 concentration was significantly lower compared to control at the three time points. In comparison with control, the lowest H_2O_2 level was recorded at 120 min when 4 mg/ml of VitB12 was added (3.13 µM vs 52.1 µM). In clear contrast to the control status, no significant differences ($P>0.05$) were noted between the different time points for each B12 treatments.



Different letters (A-C) between control and each B12 concentration at each time point denote significant difference ($P<0.05$). Different letters (a-c) between time points at each concentration denote significant difference ($P<0.05$)

Figure 1. Generation of hydrogen peroxide (H_2O_2; mean ± SD) by fresh ram spermatozoa during 120 min of incubation in tris based medium at 37 °C. Ram spermatozoa were incubated in a 96-well microplate at concentration of 10 x 10^6 spermatozoa/well

Figure 2 shows the formation level of hydrogen peroxide from chilled ram spermatozoa after 24 hours of incubation in tris egg-yolk medium at 5 °C. The rates of H_2O_2 generation were significantly ($P<0.05$) lower than control for the three concentrations of VitB12. For this type of spermatozoa the 4 mg/ml of B12 concentration produced the lowest H_2O_2 level (6.4 µM vs 64.98 µM).



Different letters (A-C) between control and each B12 concentration denote significant difference ($P<0.05$)

Figure 2. Generation of hydrogen peroxide (H_2O_2; mean ± SD) by chilled ram spermatozoa. After 24 hours of incubation in Tris – egg yolk medium at 5 °C, chilled ram spermatozoa were incubated in a 96-well microplate at concentration of 10 x 10^6 spermatozoa/well

The effects of VitB12 supplementations on CASA motion characteristics of fresh sperm are depicted in Table 2. Tris based medium supplemented with 1, and 2 mg/mL of VitB12 led to higher levels of motility percentages (MOT %), the percentages of progressive

Table 2. Effects of vitamin B12 (VitB12) on CASA sperm motion characteristics of fresh spermatozoa from five rams incubated in tris based medium at 37 °C for 120 minutes. Mean (± Sd) of CASA parameters for percent motility spermatozoa (MOT %), sperm showing progressive motility (PMOT %), average path velocity (VAP), percent straightness (STR %) as well as percent linearity (LIN %)

CASA parameter/ treatments	MOT (%)	PMOT (%)	VAP (µm/s)	STR (%)	LIN (%)
Control	82.67 ±4.53[a]	18.78±1.64[a]	116.22± 10.40[a]	61.44±3.09[a]	34.55±3.46[a]
+1 mg/ml VitB12	89.22±1.85[b]	20.44±1.74[b]	121.44±13.50[b]	61.89±1.05[a]	33.88±1.83[a]
+2 mg/ml VitB12	89.11± 4.31[b]	20.55±1.42[b]	123.89±10.10[b]	62.55±2.34[a]	35.11±2.31[a]
+4 mg/ml VitB12	73.33± 4.79[c]	14.77±2.99[c]	98.11±4.22[c]	59.55±2.60[a]	33.33±2.35[a]

Values with different superscript letters (a, c) within columns differ significantly ($P<0.05$)

Table 3. Effects of vitamin B12 (VitB12) on CASA sperm motion characteristics of chilled spermatozoa from five rams incubated in tris-egg -yolk medium at 5 °C for 24 hours. Mean (± Sd) of CASA parameters for percent motility spermatozoa (MOT %), sperm showing progressive motility (PMOT %), average path velocity (VAP), percent straightness (STR %) as well as percent linearity (LIN %)

CASA parameter/ treatments	MOT (%)	PMOT (%)	VAP (µm/s)	STR (%)	LIN (%)
Control	±79.224.43[a]	13.22±4.23[a]	93.33±10.39[a]	60.66±2.59[a]	35.66±3.12[a]
+1 mg/ml VitB12	89.78±5.17[b]	17.66±5.63[b]	104.44±18.80[b]	61.33±3.70[a]	36.22±4.32[a]
+2 mg/ml VitB12	86.22±7.41[b]	17.11±5.32[b]	99.77±21.10[b]	63.88±2.20[a]	37.66±2.12[a]
+4 mg/ml VitB12	69.22±7.79[c]	10.33±3.29[c]	82.77±13.20[c]	63.22±2.33[a]	37.77±1.85[a]

Values with different superscript letters (a, c) within columns differ significantly ($P<0.05$)

motility (PMOT %) and the average path velocity (VAP) in comparison to control group ($P<0.01$). In contrast, when VitB12 concentration was increased to 4 mg/ml, the same previous sperm motion characteristics significantly decreased ($P<0.01$). However, no differences ($P>0.05$) were noted between the different VitB12 treatments and the control group for STR % as well as for LIN %. Also, no significant differences were seen between 1 mg/ml and 2 mg/ml concentrations for the MOT %, PMOT %, VAP, STR% and LIN % after two hours of incubation at 37 °C.

Table 3 illustrates the effects of VitB12 supplementations on CASA motion characteristics of chilled sperm assessed in tris-egg yolk medium. Apart from the 4 mg/mL level and compared to the control, sperm motion characteristics including MOT %, PMOT % and VAP were significantly improved in the presence of 1 and 2 mg/ml of VitB12. Anyhow, as it was the also case for fresh samples, no differences ($P>0.05$) were noted between the different B12 treatments and the control group for STR % as well as for LIN %.

DISCUSSION

In the present study we analyzed the ability of VitB12 to change the level of hydrogen peroxide generated from ram spermatozoa and also the motility status of spermatozoa incubated with different concentrations of this vitamin. The three concentrations of VitB12 used in our study were all able to significantly decrease H_2O_2 formation from both fresh and chilled spermatozoa with a clear ability to increase or decrease motility status. Thus, these results clearly confirm the antioxidant ability of VitB12. In humans, a positive correlation between

VitB12 concentration in the seminal plasma and sperm concentration was previously noted; where this vitamin was able to reduce ROS level (Chen et al., 2001). It was also obvious from the present work that VitB12 effects in reducing H_2O_2 formation were related to the used concentration. In fact, when optimal concentration of VitB12 was added, this vitamin was able to prevent the generation of active forms of oxygen and to scavenge against ROS in bovine semen extenders (Hu et al., 2011).

We previously monitored the kinetics of H_2O_2 formation from live and dead bull, buck, and ram spermatozoa (Alomar and Donnay, 2006; Alomar et al., 2016; Alomar, 2018). Moreover, the formation rates of this ROS agent were related to spermatozoa types, spermatozoa concentration and the content of incubation media (Alomar 2018; 2019). Catalase was the most effective antioxidant system in inhibiting H_2O_2 generation in *in vitro* condition (Ball et al., 2001; Alomar et al., 2016). VitB12 appears to be a very useful compound in neutralizing this ROS element. In the future, one of the interesting experiments that could be conducted is to use a mixture of both catalase and VitB12 to achieve a better control on hydrogen peroxide formation from ram spermatozoa. Nevertheless, further studies must be carried out to explore the possibility of using a combination of different antioxidants in various doses to install ideal preservation systems for sheep semen.

Several key mechanisms may explain the antioxidant ability of VitB12. According to Van de Lagemaat et al. (2019) the potential antioxidant properties of VitB12 include: scavenging of ROS by preservation of glutathione and reduction of homocysteine-induced oxidative stress. Furthermore, supplementations of frozen ram spermatozoa by VitB12 increased the effectiveness of antioxidant enzymes glutathione peroxidase (GPx) and superoxide dismutase (SOD), which were able to reduce the oxidative stress (Asadpour et al., 2012). According to Asadpour et al. 2012, VitB12 could protect sperm membranes and their capacity to protect spermatozoa from stress induced by cold widely varied with the used concentration.

VitB12 may indirectly stimulate ROS scavenging by the preservation of glutathione, which involves an intricate network of reactions that has not been fully elucidated (Manzanres and Haroly, 2010; Karamshettyet al., 2016). On the other hand, vitamins B6 and B12 were important cofactors in homocysteine (Hcy) metabolism (Green et al., 2017). VitB12 stimulates the conversion of homocysteine to methionine, and its deficiency caused an important increase of Hcy levels (Green et al., 2017). Increased levels of Hcyhave been closely related to oxidative stress and it is believed that Hcy mediates ROS accumulation through multiple mechanisms, e.g., Hcy auto-oxidation leading to production of H_2O_2 (Lascalzo, 1996; Tyagi et al., 2005).

Two tris media were used in this study including tris based medium for the fresh samples and tris-egg-yolk medium for the chilled ones. It is well known that tris medium is widely available, promotes high sperm motility and is a particle-free medium that has low viscosity. This basic medium has several advantages for routine evaluation of fresh semen and when liquid ram semen was stored at 5 and 20 °C in this mediumit was better over time for motility characterisitics, membrane integrity and viability than both sodium citrate and milk-based media (Paulenz et al., 2002). Tris plus egg - yolk has been widely used for the artificial insemination in different animal species including the ram (Salamon and Maxwell, 1995a; 1995b). These two tris media have been in use in our laboratory for several years and the motility values of rams and bucks spermatozoa incubated in these media were previusly compared for CASA analysis (Alomar, 2018; Alomar et al. 2018b). Nevertheless, the present results clearly show that VitB12 supplementation, depending on the concentration used, could significantly enhance the status of spermatoza preserved in tris media.

In both humans and animals, CASA has proved its usefulness in assessing motility parameters (Larsen et al., 2000; Verstegenet al., 2002) and in studying the variations in semen parameters within and between donors (Wijchmanet al., 2001). The motility results of this study confirmed that the addition of 1 and 2 mg/ml of

VitB12 to both fresh and chilled samples led to significant increase of percentage of motile sperms, percentage of progressive sperms and also velocity values. In agreement to our results, the addition of 2.50 mg/mL of VitB12 into freezing media increased the movement characteristics of bovine spermatozoa (Hu et al., 2009). Furthermore, the use of VitB12 in goat semen extenders significantly improved important parameters of CASA motility kinetics of spermatozoa compared to controls containing low-density lipoproteins (Shaltaf et al., 2019). The results of Hamedani et al. (2013) indicated that the addition of 2 mg/ml of VitB12 to tris extender for Dallagh rams increased their motility, progressive motility, viability and the number of normal spermatozoa. By using Awassi ram spermatozoa, Saieed and co-workers (2018) showed that adding 0.5 or 1 µg of VitB12 with nitrogen vapour technique enhanced several sperm parameters post- thawing inculding motility characteristics. In the present study we used the semen of the same sheep species as in the previous report, but here we introduced other spermatozoa types including the fresh and chilled ones. In fact, such experimental works using different spermatozoa types including frozen-thawed; fresh or chilled could largely help in enhancing spermatozoa preservation status of this local sheep species.

In our work, MOT %, PMOT % and VAP were the most important CASA measurements which showed clear differences between the controls of fresh and chilled samples and the different treatments groups of VitB12. In contrast, the values of STR % and LIN % parameters did not show any obvious differences. It must be noted that VAP is the most important sperm velocity parameter in CASA system, while LIN % and STR % describe sperm trajectory. Thus, B12 has affected the percentage of motile spermatozoa and spermatozoa velocity but not their trajectory. We previously showed that MOT %, PMOT % and VAP parameters of Awassi motile spermatozoa incubated in different semen media were significantly affected by the osmolality and pH levels of the media, while the values of both LIN % and STR % were not different from those of control samples (Alomar et al., 2018b).

Despite the positive effect of the 4 mg/ml level in reducing H_2O_2, this concentration had a negative influence on the motility for both fresh and chilled samples. The dosage of the antioxidant is a critical point, but the investigators do not know the exact required level of ROS for physiological purposes (Agarwal and Prabakaran, 2005). Indeed, an excessive addition of VitB12 to the semen media can neutralize the oxidative stress induced by the high ROS concentration, but at the same time it can stop the normal sperm functions. High concentration of VitB12 had a toxic effect on bull spermatozoa (Hu et al., 2011). However, the exact mechanism of the negative effects of high doses of this vitamin on spermatozoa characteristics requires further investigations.

CONCLUSION

In the present study the effects of VitB12 on hydrogen peroxide generation and motility characteristics of fresh and chilled ram spermatozoa were investigated. It can be concluded that VitB12 had a clear antioxidant capacity. For the first time the antioxidant capacity of VitB12 was proved by showing its ability in reducing H_2O_2 generation from ram spermatozoa. Our data clearly showed that VitB12 seem to be promising antioxidant choice for ram semen media and a motility stimulant which could preserve and enhance fresh and chilled sheep spermatozoa.

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