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Effects of hydrogen peroxide and oxidase substrates on ram sperm motility

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Additional keywords

Reactive oxygen species.
Awassi.
CASA.

SUMMARY

In this study, the effects of adding hydrogen peroxide (H_2O_2), nicotinamide adenine dinucleotide phosphate (NADPH) and phenylalanine (substrates of specific oxidases) on ram sperm motility were investigated. Fresh Awassi ram sperm were incubated at 37 °C for 30, 60, 120 and 180 minutes with (100 µM and 1 mM) or without H_2O_2, NADPH and phenylalanine in egg yolk Tris (EYT) and Tyrode albumen lactate (TAL) media. Sperm motility was assessed using computer aided sperm analysis (CASA). Following sperm incubation with 100 µM of H_2O_2 at different time intervals, no significant changes (P>0.05) were observed compared to control in percent of motility (MOT %), progressive motility (PMOT %) and average path velocity (VAP). Incubation with 1 mM H_2O_2 led to a significant (P<0.05) decrease in all motility parameters. Higher values (P<0.05) of CASA parameters (especially PMOT % and VAP) were achieved when 1 mM of NADPH and phenylalanine were added in the two media. We concluded that fresh Awassi ram sperm appears to support certain levels of hydrogen peroxide in both TAL and EYT media and this may reflect an antioxidant capacity. Moreover, the addition of oxidase substrates in 1 mM concentration to ram semen media could be useful as stimulating additives for ram sperm motility.

Mots-clés supplémentaires

Espèces réactives de l'oxygène.
Awassi.
CASA.

Effets des substrats de peroxyde d'hydrogène et d'oxydase sur la motilité du sperme de bélier

RÉSUMÉ

Dans cette étude, les effets de l'ajout de peroxyde d'hydrogène (H_2O_2), de nicotinamide adénine dinucléotide phosphate (NADPH) et de phénylalanine (substrats spécifiques d'oxydases) sur la mobilité des spermatozoïdes ont été étudiés. On a incubé des frais spermatozoïdes d'Awassi belier à 37 °C pendant 30, 60, 120 et 180 minutes avec (100 uM et 1 mM) ou sans H_2O_2, NADPH et phénylalanine dans des milieux de Tris (EYT) et de Tyrode Albumen Lactate (TAL). La mobilité des spermatozoïdes a été évaluée à l'aide d'une analyse du sperme assistée par ordinateur (CASA). Après l'incubation des spermatozoïdes avec 100 µM de H_2O_2 aux différents intervalles de temps, aucun changement significatif (P>0,05) n'a pas été observé comparé au contrôle par le pourcentage de mobilité (MOT%), la mobilité progressive (PMOT%) et la vitesse moyenne (VAP). L'incubation avec 1 mM de H_2O_2 a entraîné une diminution significative (P<0,05) de tous les paramètres de mobilité. Des valeurs supérieurs (P<0,05) des paramèters CASA(en particulier PMOT% et VAP) ont été obtenues lorsque 1 mM de NADPH et de phénylalanine ont été ajoutés dans les deux milieux. Nous avons conclu que les frais spermatozoïdes d'Awassi bélier semblent supporter certains niveaux de peroxyde d'hydrogène dans les milieux TAL et EYT et cela peut refléter une capacité anti-oxydante. L'addition de substrats d'oxydase d'une concentration de 1 mM à des milieux de semence de bélier pourrait être utile en tant qu'additifs de stimulation pour mobilité de spermatozoïdes de bélier.

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INTRODUCTION

Sperm motility is a critical factor in determining semen quality and fertilizing ability (Holt et al. 2007). It is well known that several factors including temperature, osmolality, pH, and the used medium may affect sperm motility. Reactive oxygen species (ROS) have also been correlated to decreased motility parameters, consequently, having adverse effects on fertility (Saleh and Agarwal 2002). The ROS family members include superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($HO^{\cdot}$), and hydrogen peroxide (H_2O_2) which are generally derived from the incomplete reduction of molecular oxygen. It is worth noting that hydrogen peroxide is relatively a stable agent and

has a high oxidant potential and can freely cross cell membranes (Halliwell and Gutteridge 1989). Moreover, H_2O_2 is the main ROS responsible for oxidative damage to the sperm (Alvarez et al. 1987) with high ability to impair the plasma and mitochondrial membranes and to decrease the metabolic activity required for the maintenance of sperm motility. In contrast to its negative role in semen, evidences indicate a positive role of H_2O_2 in a variety of sperm functions, including acrosome reaction, capacitation and hyperactivated motility. Hydrogen peroxide and superoxide anion play a role in the regulation of sperm capacitation by inducing protein tyrosine phosphorylation in human (De Lamirande and Lamothe 2009) and bull (Rivlin et al. 2004).

The mechanism of hydrogen peroxide production by spermatozoa is a matter of much controversy. However, sperm cells may generate ROS by different ways including nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase system at the level of sperm membrane (Aitken et al 1992; Rivlin et al. 2004), NADH-dependent oxido- reductase diphorase in the mitochondria (Gavella and Lipovac 1992). Moreover, a specific aromatic amino acid oxidase (AAAO) has been identified as the origin of ROS formation from dead bovine and ram sperm (Shannon and Curson 1982a; Shannon and Curson 1982b; Upreti et al. 1998). We previously found that the addition of NADPH and phenylalanine (the substrates for NADPH-oxidase and AAAO, respectively), had a significant effects on the production of H_2O_2 from the both live and dead bull (Alomar and Donnay, 2006) and ram sperm (Alomar et al. 2016).

Several artificial insemination (AI) and *in vitro* fertilization (IVF) media have been designed, to protect and maintain spermatozoa during the processing and storage of the semen. Tris plus egg yolk have been widely used for AI by using fresh or cryopreserved sperm (Salamon and Maxwell, 1995). On the other hand, most IVF labs use Tryode Albumin Lactate Pyruvat (TALP) medium for *in vitro* fertilization experiments (Farrell et al. 1996). Moreover, the used AI and IVF media and extenders should also provide an environment that inhibits the formation of harmful reactive oxygen species or lipid peroxide (Orok et al. 2010). Despite the existence of different studies concerning sperm motility in AI and IVF media reported in the literature, there are no clear evidences available showing which media are the most efficient to sustain sperm motility under stress conditions.

The adverse effects of H_2O_2 on sperm motility exemplify the importance of minimizing the introduction of this ROS agent in different semen media used in *in vivo* and *in vitro* experiments. Moreover, the effects of addition of NADPH and phenylalanine on ram sperm motility have not been previously studied. Thus, the present study focused on the effects of the addition of H_2O_2 and these two specific substrates on fresh Awassi ram sperm motility assessed by CASA technology.

MATERIALS AND METHODS

Semen collection and processing

This study was carried out at Der-Al-Hajar Animal Production Research Station, 33 km south-east of Damascus. Semen was obtained from ten sexually-experienced Awassi rams, aged between 3 and 4 years. Semen was collected with the aid of an electro-ejaculator (Minitube - Electro Ejaculator, Tiefenbach, Germany) administrating a series of 32 cycles of short electrical stimuli with each cycle delivering a slightly higher intensity. It must be noted that all used animals were under veterinary care supervision after semen collection and animal's welfare were highly respected. Moreover, the experiments for this study were approved by the Local Scientific and Ethical Committee of the Atomic Energy Commission of Syria (AECS), Damascus, Syria (permit number 68-2014). Upon collection, the semen was immediately evaluated for its general appearance and volume. Sperm concentration was estimated using a haematocytometer. An initial analysis of sperm motility was performed using CASA system. Sperm samples with a motility score ≥ 75 % of motile sperm and a concentration of ≥ 1 x 10^9 spermatozoa/mL were employed.

Experimental design and media preparation

Three experiments were conducted with semen collected from ten different rams and in each experiment, a total of 30 ejaculates were used. Ten ejaculates were mixed in each replicate to isolate the individual effect of the ten males, and each experiment was repeated for three times. In all the experiments, spermatozoa were incubated in tubes in a water bath at a concentration of 25 x 10^6 sperm/ml in a final volume of 500 µL of egg yolk Tris (EYT) and Tyrode Albumin Lactate (TAL) media at 37 ºC in air for 3h. The EYT medium 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 80 ml of distilled water, plus 20 ml of egg yolk, bringing the total volume to 100 ml. The TAL solution prepared as a 300 mOsm/Kg solution contained the following in g/L $^{-1}$: 6.6 g NaCl, 0.24 g KCl, 0.04 g NaH_2PO_4, 1.92 g $NaHCO_3$, 0.1 g $MgCl_2.6H_2O$, 0.3 g $CaCl_2.2H_2O$, 0.5 g $C_3H_5NaO_3$ and 6 g bovine serum albumin. The two media components were held constant at pH 7. The first experiment was designed to examine the effects of two concentrations (100 µM and 1 mM) of hydrogen peroxide on ram sperm motility diluted TAL and EYT compared to control (untreated semen) at 30, 60, 120 and 180 minutes of incubation. The second experiment was conducted to examine the effects of two levels of NADPH (100 µM and 1 mM) compared to control on sperm motility diluted in the same two previous media at 30, 60, 120 and 180 minutes of incubation. Sperm motility was assessed in the third experiment at 30, 60, 120 and 180 minutes of incubation in TAL and EYT containing 0, 100 µM, 1 mM of phenylalanine. It must be noted that several concentrations of H_2O_2, NADPH and phenylalanine (100 µM, 200 µM, 500 µM, 1 mM and 2 mM)

were tested before the conduction of the final experiments of the present work; the findings showed that the 100 µM and the 1 mM concentrations were the most appropriate to show the effects of those concentrations on sperm motility (unpublished data).

Assessment of sperm motility

The motility characteristics of the sperm were assessed by CASA technique, using the Hamilton-Thorne motility analyzer (Hamilton Thorne Biosciences, HTM version 12.3, Beverly, USA). Five microliters aliquots of diluted semen were placed in the system lame (dual sided sperm analysis chamber, depth 20 µm, Hamilton Thorne Biosciences) and loaded into the analyzer. For each sperm sample, three fields were selected and counted at random and assessed to generate data from at least 200-250 sperm/sample. The motility characteristics included in the analysis were: the percent motility (MOT %), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s) and the percent of sperm showing progressive motility (PMOT %: VAP ≥ 75 µm/s and STR ≥ 80 %). The HTM system settings of ovine spermatozoa are presented in **Table I**.

Statistical analysis

Statistical analysis was conducted with the Minitab program (Minitab Coventry, United Kingdom, Version 13.31, 2000). The normality of values distribution was first tested with the Shapiro-Wilk test. Data regarding hydrogen peroxide, phenylalanine and NADPH were subjected to a factorial analysis of variance for the three concentrations at each time point (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 the effects of different concentrations of H_2O_2 on sperm motility in both EYT and TAL media. In absence of hydrogen peroxide and at the start of incubation, sperm percent motility was between 94 % and 87 % in EYT and TAL media, respectively. MOT % decreased significantly ($P<0.05$) to 85 % in EYT medium and to 73 % in TAL after 3 h of incubation. The progressive sperm motility and mean path velocity were maintained for 3 h to averages of 22 % and 19 % for PMOT % and 98 µm/s and 87 µm/s for VAP in both EYT and TAL media, respectively. For the two media and the three experiments, the profile of VCL and VSL velocity parameters was exactly the same as VAP with the same significant indicators. No significant changes ($P>0.05$) in MOT %, PMOT % and VAP parameters were observed following their incubation with 100 µM H_2O_2 at the different time intervals in the two media. The significant damaging effect ($P < 0.05$) of 1 mM H_2O_2 on all CASA sperm motility parameters in both EYT and TAL occurred within 30 min. After 3 h of incubation MOT %, PMOT % and VAP drooped significantly to 45 % , 5 % and 52 µm/s in EYT media and to 29 %, 2 % and 35 µm/s in TAL, respectively.

The effect of NADPH on CASA motility parameters during the different incubation periods is illustrated in **Figure 2**. No significant differences ($P>0.05$) were observed in motility parameters (MOT %, PMOT %, VAP, VCL, VSL) after exposure to 100 µM of NADPH. Addition of 1 mM of NADPH for 3 h of incubation in both media had a significant positive effect ($P < 0.05$) on motility parameters (PMOT %,VAP, VCL and VSL).

Figure 3 shows the motility of sperm treated with 0, 100 µM and 1 mM of phenylalanine in both EYT and TAL media during 30, 60, 120 and 180 minutes of incubation. A concentration of 100 µM of phenylalanine did not significantly ($P>0.05$) affect MOT %, PMOT % and VAP compared to control. It was found that 1 mM of phenylalanine significantly ($P < 0.05$) increased PMOT %, VAP, VCL and VSL parameters in the two media.

Table I. The settings for the Hamilton Thorne Biosciences system version 12.3 used to evaluate ram semen (Les paramètres pour le système Hamilton Thorne Biosciences version 12,3 utilisé pour évaluer le sperme de bélier).

Parameter	Setting
Frame rate (Hz)	60
Frames acquired (no)	30
Minimum contrast	60
Minimum cell size (pixels)	5
Low VAP cut off	21.9
Low 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)	0.25/1.50

DISCUSSION

Despite the effect of hydrogen peroxide addition on sperm motility of different species including the ram had been previously studied (Bilodeau et al. 2002; Garg et al. 2009; Maia et al. 2014), this is the first study shows the effect of this ROS agent with the two special substrates responsible for H_2O_2 production from fresh Awassi sperm. Our results clearly contrast with those reported by Maia et al. (2014) study whereas a 100 µM concentration of H_2O_2 was sufficient to negatively affect sperm motility of Santa Inês ram. It must be noted that the difference between our study and the previous one is that Maia and co-workers conducted their experiments using frozen-thawed samples. Freezing and thawing process may partly impair the scavenging mechanism in thawed sperm compared to fresh ones. Furthermore, cryopreserved sperm are more sensitive to lipid peroxidation resulted from hydrogen peroxide incubation and higher malondialdehyde levels were reported in cold shocked and frozen-thawed spermatozoa (Kumaresan et al. 2006). In buffalo, the decrease of sperm functions with duration of incubation and concentration of H_2O_2 was significantly higher in frozen–thawed than fresh and equilibrated spermatozoa (Garg et al. 2009). However, the difference between

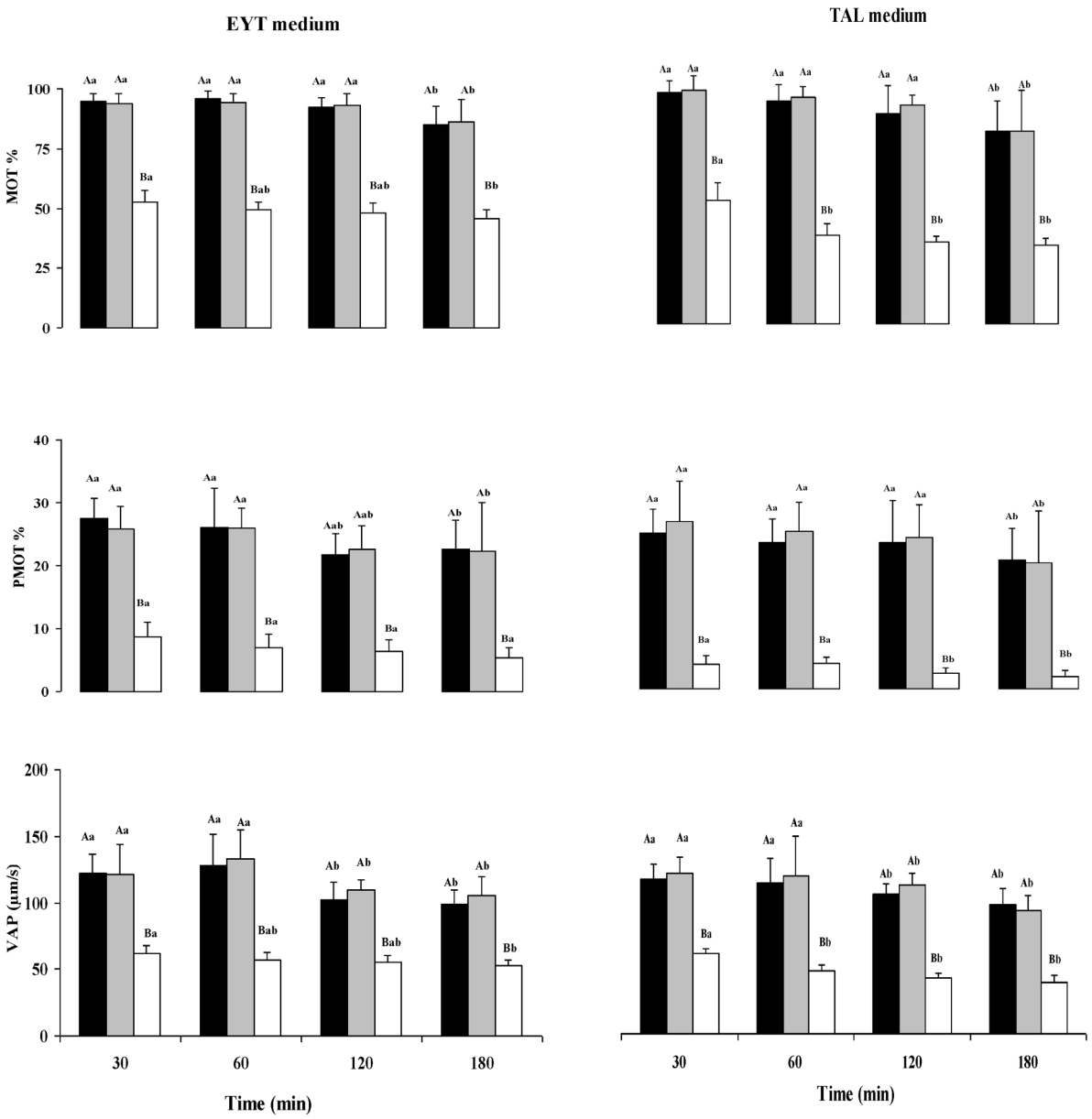

Figure 1. Effects of H_2O_2 on percent motility, percent of sperm showing progressive motility and average path velocity in EYT and TAL media (■) control, (■) semen treated with 100 µM H_2O_2, (□) semen treated with 1 mM of H_2O_2 (Effets de H_2O_2 sur le pourcentage de mobilité de sperme, le pourcentage de sperme affichant une mobilité progressive et la vitesse moyenne dans les milieux EYT et TAL (■) contrôle des milieux, (■) sperme traité avec 100 µM de H_2O_2, (□) sperme traité avec 1 mM H_2O_2). Data are mean ± SD of three replications. A-C Different letters in each medium within the same time point denote significant difference (ANOVA, $P < 0.05$). [a-c]Different letters in each medium between the different time points denote significant difference (ANOVA, $P<0.05$).

the semen of different species and between different breeds concerning the ability to sustain stress conditions could not be excluded. We previously observed a high flexibility of fresh Awassi ram sperm in adjusting different stress conditions at different osmolality, pH and temperature levels (Alomar et al. submitted).

CASA parameters showed an obvious negative effect when sperm were incubated at 1 mM concentration of H_2O_2. It has been suggested that the inhibition of sperm motility after incubation with ROS was caused by a depletion of ATP and a decrease in axonemal protein phosphorylation (De Lamirande and Gagnon 1995). In spite of that 1 mM concentration could be considered a very high ROS level, the decrease in motility rates in this study was not as pronounced as in other reports (Bilodeau et al. 2002; Maia et al. 2014), and this may reflect an antioxidant capacity of fresh ram sperm. Anyhow, additional researches can be performed on assessing the effects of other H_2O_2 concentrations and also other types of ROS family (eg. Superoxide anion and hydroxyl radical) on ram sperm motility.

Tyrode Albumin Lactate Pyruvate (TALP) medium is a well characterized physiological solution and one

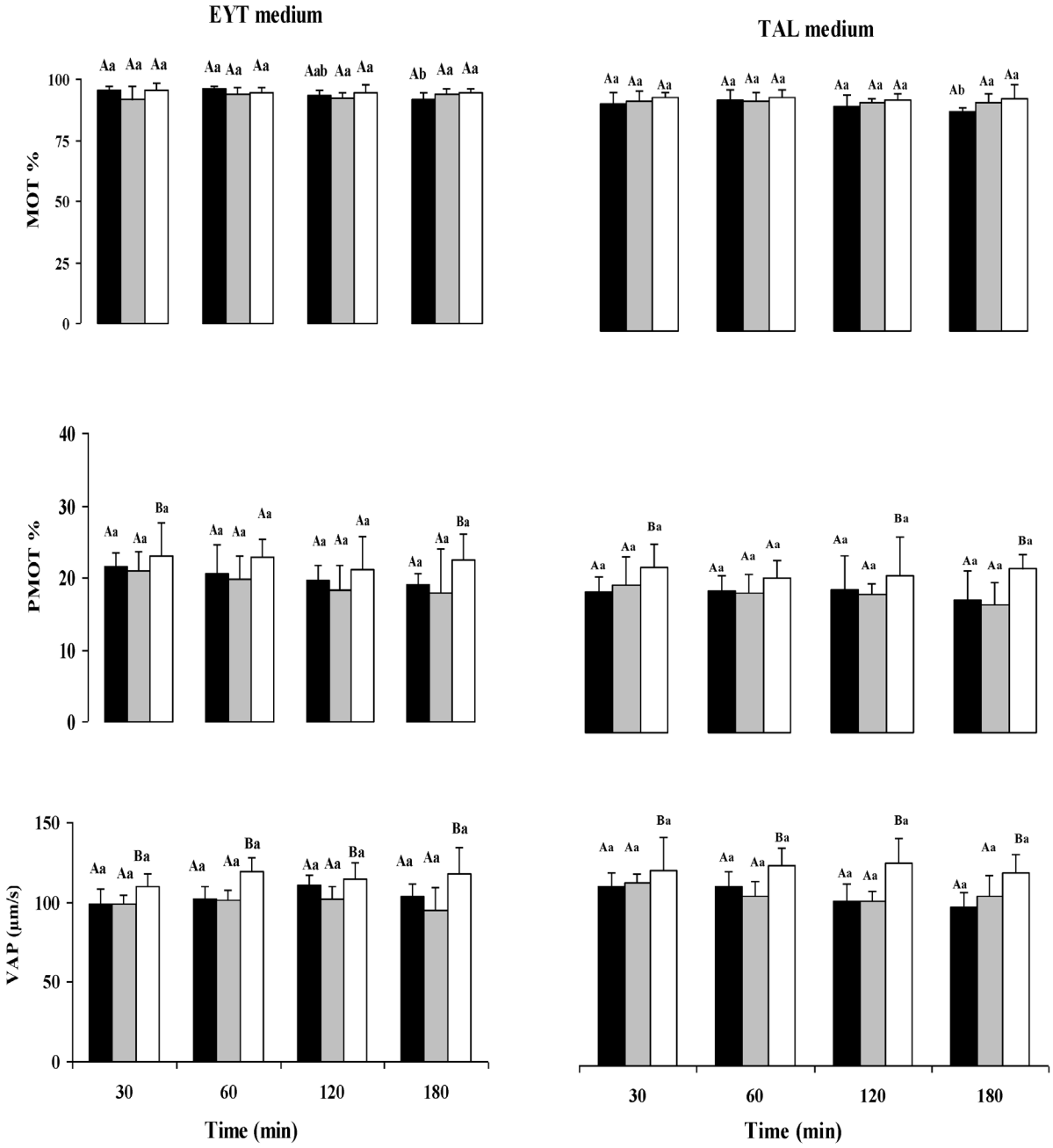

Figure 2. Effects of NADPH on percent motility, percent of sperm showing progressive motility and average path velocity in EYT and TAL media. media (■) control, (■) semen treated with 100 µM NADPH, (□) semen treated with 1 mM of NADPH (Effets de la NADPH sur le pourcentage de mobilité de sperme, le pourcentage de sperme affichant une mobilité progressive et la vitesse moyenne dans les milieux EYT et TAL. (■) contrôle des milieux, (■) sperme traité avec 100 µM de NADPH, (□) sperme traité avec 1 mM NADPH). . Data are mean ± SD of three replications. $^{A-C}$Different letters in each medium within the same time point denote significant difference (ANOVA, $P < 0.05$). $^{a-c}$Different letters in each medium between the different time points denote significant difference (ANOVA, $P<0.05$).

of the most important fertilization media used in *in vitro* experiments (Farrell et al. 1996). Pyruvate was removed from the TALP medium throughout the present study. Pyruvate contained in TALP has been reported to have efficient antioxidant properties in semen diluent (Uperti et al. 1998). Our results contrast with Bilodeau and co-workers (2002) as they noted that bull sperm motility was reduced significantly 20-fold lower in EYT than in TALP medium. As in Maia et al. (2014) using ram sperm, Bilodeau and co-worker used frozen pool of bull semen rather than fresh semen. On the other hand, it has been reported that lipoproteins contained in egg yolk were shown to have antioxidant properties (Yamamoto and Omori, 1994). Moreover,

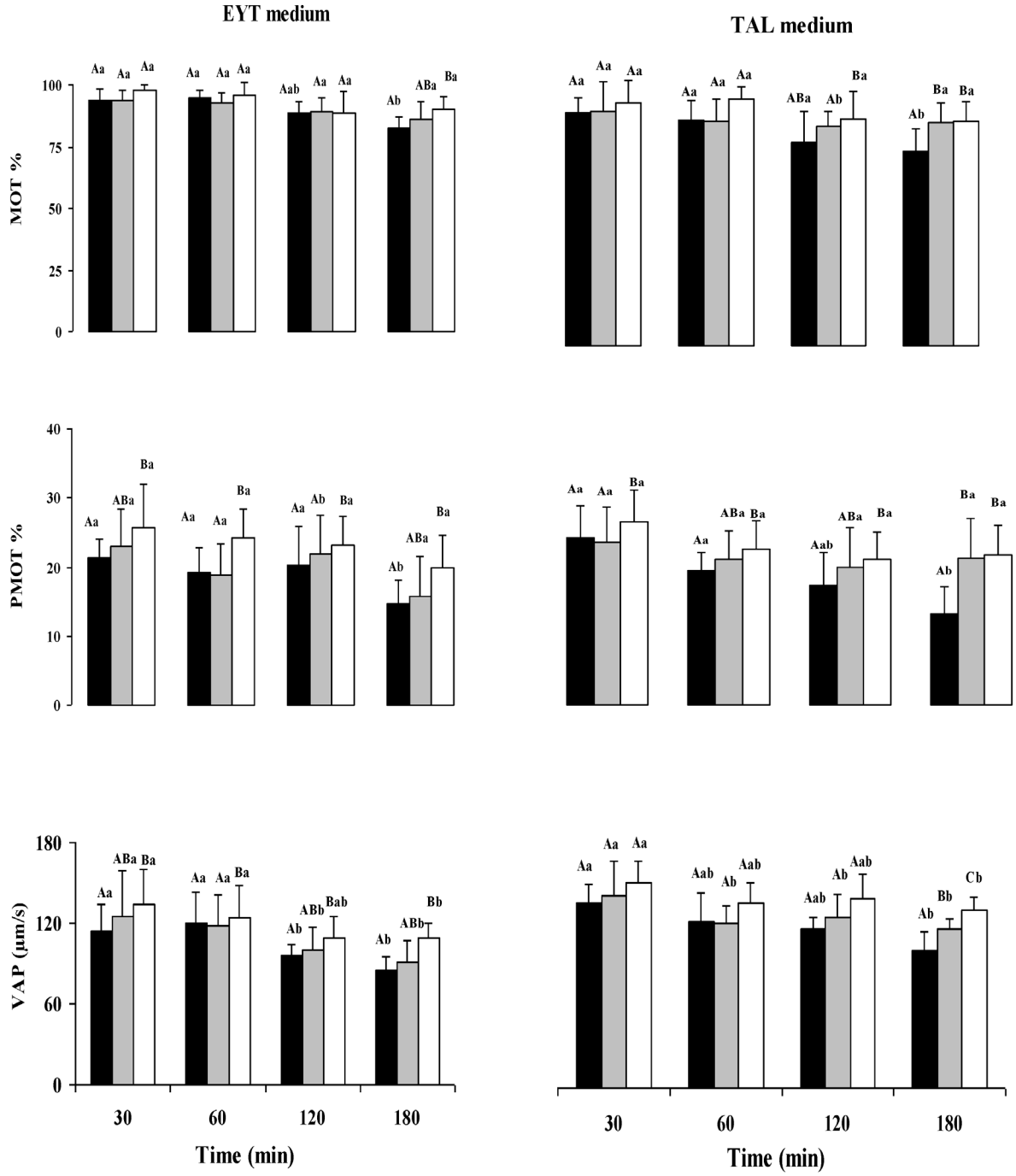

Figure 2. Effects of NADPH on percent motility, percent of sperm showing progressive motility and average path velocity in EYT and TAL media. media (■) control, (■) semen treated with 100 µM NADPH, (□) semen treated with 1 mM of phenylalanine (Effets de la NADPH sur la motilité pour cent, pour cent du sperme montrant la motilité progressive et la vitesse moyenne de chemin dans les médias AET et TAL. contrôle des milieux (■), (■) sperme traité avec 100 µM de Phénylalanine, (□) sperme traité avec 1 mm de Phénylalanine). Data are mean ± SD of three replications. $^{A-C}$ Different letters in each medium within the same time point denote significant difference (ANOVA, $P < 0.05$). a-c Different letters in each medium between the different time points denote significant difference (ANOVA, $P<0.05$).

egg yolk contains no enzymatic antioxidants such as glutathione peroxidase, α- tocopherol and carotenoids (Wilson et al. 1992). If those non-enzymatic antioxidants were not degenerated, this could refer to an antioxidant capacity of EYT which could be responsible for the sustained ability of ram sperm motility.

In equine and bull spermatozoa, NADPH increased H_2O_2 generation as detected by the Amplex Red assay (Ball et al. 2001; Alomar and Donnay 2006). Considera-

ble and significant rates of H_2O_2 were generated from live and dead ram sperm supplemented with NADPH (Alomar et al. 2016). It was obvious that when 100 µM of NADPH was used, ram sperm motility was not negatively affected, while 1 mM of this substrate positively increased both PMOT % and VAP parameters. This result could be attributed to controlled amounts of H_2O_2 produced by sperm following NADPH addition. In this respect, when buffalo spermatozoa were incubated with NADPH, capacitation was increased significantly compared to control (Roy and Atreja 2008). It must be noted that NADPH serves as a source of electrons for the generation of ROS via a proposed NADPH-oxidase reaction in spermatozoa (Aitken 1997), and this substrate appears to be implicated in sperm capacitation (O'Flahertly et al. 2006).

Phenylalanine is considered one of the major substrates of the AAAO enzyme. Hydrogen peroxide was generated after phenylalanine addition to bull and ram sperm showing AAAO activity in the spermatozoa of these species (Shannon and Curson 1982a; Alomar et al. 2016). This oxidase could be located in the tail of bovine sperm, and its activity was detected only after sperm death (Shannon and Curson 1982a). Lapointe and Sirard (1998) reported that phenylalanine had significant negative influence on bovine sperm motility and this was related to hydrogen peroxide effect. In a contrast result to previous study, phenylalanine had no negative effects on CASA parameters and 1 mM of it was able to increase ram sperm velocities. It should be noticed that in ram and bull spermatozoa, the activity of AAAO was influenced by temperature, pH and the components of the assay medium (Shannon and Curson, 1972; Upreti et al. 1998). Thus, several factors need to be considered in evaluating the activity of AAAO and its relation to ram sperm motility. Moreover, further researches are needed to fully establish the level at which the specific substrates NADPH and phenylalanine are necessary for optimizing ram sperm motility.

CONCLUSION

Fresh Awassi ram sperm appear to support a level of hydrogen peroxide as high as 100 µM in both TAL and EYT media. Such result may refer to antioxidant ability in fresh ram sperm. The addition of NADPH and phenylalanine did not negatively affect ram sperm motility and a concentration of 1 mM of both substrates may optimize sperm velocities. Thus, addition of these oxidase substrates to semen media should be seriously considered and could be used as stimulating sperm motility additives.

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