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EFFECTS OF OXIDASE SUBSTRATES ON THE CHARACTERISTICS OF GOAT SPERMATOZOA MOTILITY: SHORT COMMUNICATION

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ABSTRACT

In this study, the effects of nicotinamide adenine dinucleotide phosphate (NADPH) and phenylalanine on goat sperm motility were investigated. Fresh Shami goat semen was collected and incubated at 37 °C for 45 minutes in Tris-based medium with 1, 2 and 4 mM or without both NADPH and phenylalanine. Sperm motility was assessed using computer assisted sperm analyzer (CASA). Higher velocity values were achieved when 1 and 2 mM of NADPH were added, while the addition of phenylalanine at the same concentrations had reduced the values of all motility parameters, with no significant differences ($P > 0.05$) compared to the control. Addition of the two substrates at a concentration of 4 mM had a clear negative effect ($P < 0.05$) on the values of sperm motility parameters as well as on the percentages of spermatozoa subpopulations, especially for the rapid and the static categories. We concluded that both NADPH and phenylalanine have pronounced effects on goat sperm motility and these effects were dependent on concentration. The supplementation of semen media with NADPH at an appropriate concentration can be useful as a stimulating additive for goat sperm motility.

Key words: hydrogen peroxide; phenylalanine, NADPH, goat, sperm motility

INTRODUCTION

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydroxyl radical ($OH^\cdot$) and hydrogen peroxide (H_2O_2) are chemically reactive molecules resulting from oxygen consumption. In general, ROS family members play an important role in normal sperm function (Ford, 2004), for example, H_2O_2 participates in the regulation of sperm capacitation process by inducing protein tyrosine phosphorylation in human (De Lamirande and Lamothe, 2009), equine (Baumber *et al*., 2000) and bull (Rivlin *et al*., 2004). In contrast, elevated ROS concentrations resulting from an imbalance in the production of ROS and antioxidant systems may adversely affect spermatozoa motility, membrane integrity and IVF outcome (Du Plessis *et al*., 2008).

Spermatozoa may generate ROS through nicotinamide adenine dinucleotide phosphate NADPH-oxidase system located in the plasma membrane (Aitken *et al*., 1992; Rivlin *et al*. 2004). On the other hand, a specific aromatic amino acid oxidase (AAAO) has been identified as the origin of ROS formation from dead sperm (Shannon and Curson, 1982a; 1982b; Upreti *et al*., 1998). We previously showed that the addition of NADPH and phenylalanine (the substrates for NADPH-oxidase and AAAO, respectively), had significantly raised H_2O_2 formation from live and dead bull (Alomar and Donnay, 2006), ram (Alomar *et al*., 2016) and buck sperm (Alomar, 2018). Furthermore, when ram spermatozoa where supplemented with these two substrates, the values of velocity parameters were significantly augmented (Alomar *et al*., 2018).

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Our previous studies clearly indicated the importance of NADPH and phenylalanine in the spermatozoa function of different ruminants' species. However, the influence of NADPH and phenylalanine on goat spermatozoa motility had not been previously studied.

Thus, the main aim of the present study was to determine the effects of the addition of these two specific oxidase substrates on goat sperm motility assessed by CASA technology.

MATERIAL AND METHODS

Semen collection

This study was carried out at Der-Al-Hajar Animal Production Research Station, 33 km south-east of Damascus. Semen was obtained from five sexually experienced Shami bucks, aged between 3 and 4 years. Semen was collected with the aid of an electro-ejaculator (Minitube – Electro Ejaculator, Tiefenbach, Germany). It must be noted that 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 36-Z/M4 – 2019).

Experimental design and medium preparation

Two experiments were conducted with semen from the five bucks and in each experiment a total of 15 ejaculates were used. The ejaculates were mixed in each replicate to isolate the individual effect of males, and each experiment was repeated for three times. Spermatozoa were incubated in tubes in a water bath at a concentration of 25 x 10^6 sperm.ml^{-1} in a final volume of 500 µL of Tris-based medium prepared as a 300 mOsmol.kg^{-1} 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. The medium components were kept constant at pH 7. The first experiment was designed to examine the effects of three concentrations of NADPH (1, 2 and 4 mM) at 37 °C after 45 minutes of incubation. The second experiment was conducted to examine the effects of three concentrations of phenylalanine (1, 2 and 4 mM), compared to control, on sperm motility at 37 °C after 45 minutes of incubation.

Motility assessment

The motility characteristics of the spermatozoa were assessed by CASA technique using the Hamilton-Thorne motility analyzer (Hamilton Thorne Biosciences, HTM version 12.3, Beverly, USA). For each sperm sample, three viewing fields were selected and counted randomly to assess the data from at least 200-250 sperm cells per sample. The motility characteristics included in the analysis were: the percent motility (MOT %), curvilinear velocity (VCL, µm.s^{-1}), average path velocity (VAP, µm.s^{-1}), straight line velocity (VSL, µm.s^{-1}) and the percent of sperm showing progressive motility (PMOT %: VAP ≥ 75 µm.s^{-1} and STR ≥ 80 %). Spermatozoa subpopulations were defined in four categories by CASA system as following. Rapid (4): fraction of all cells moving with VAP > path velocity (VAP = 25 µm.s^{-1}). Medium (3): fraction of all cells moving with VAP cutoff (5 µm.s^{-1}) < VAP < path velocity (VAP = 25 µm.s^{-1}). Slow (2): fraction of all cells moving with VAP < VAP cutoff (5 µm.s^{-1}) or VSL < VSL cutoff (11 µm.s^{-1}). Static (0-1) fraction of all cells that is not moving at all.

The HTM settings used for goat spermatozoa were negative phase contrast optics at a recording rate of 60 frame/sec, temperature of analysis – 37 °C, light adjustment – 90-110, minimum cell size – 5 pixels, non-motile head size – 5 pixels, non-motile head intensity – 55, low VAP cut off – 21.9 µm.s^{-1}, low VSL cut off – 6 µm.s^{-1}, static size limit – 0.60/8 (min/max), static intensity limit – 0.25/1.50 (min/max), static elongation – 0/95 (min/max).

Statistical analysis

Statistical analysis was conducted using the Minitab program (Minitab Coventry, United Kingdom, Version 13.31, 2000). Data regarding phenylalanine and NADPH effects on spermatozoa were subjected to a factorial analysis of variance for the three concentrations (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

Table 1 shows the effects of different NADPH concentrations on CASA parameters. Addition of 1 mM

and 2 mM of NADPH had a significant positive effect ($P < 0.05$) on motility parameters MOT %, PMOT %, VAP, VCL and VSL, while a concentration of 4 mM significantly reduced the values of MOT % VAP, VCL and VSL.

Table 2 shows the motility of sperm treated with 0, 1, 2 and 4 mM of phenylalanine during 45 minutes of incubation. Phenylalanine given at concentrations of 1 mM and 2 mM insignificantly ($P > 0.05$) reduced motility values compared to the control. When 4 mM of phenylalanine was added, a significant ($P < 0.05$) decrease in all CASA analyzed parameters was observed. Figures 1 and 2 show the effects of NADPH and phenylalanine on the distribution of goat sperm subpopulations according to the motility. The rapid and static categories were the most affected subpopulations especially when the two substrates were added at a concentration of 4 mM.

Table 1. Effects of NADPH on sperm motility parameters of goat sperm samples

CASA parameter / Treatment	MOT (%)	PMOT (%)	VAP (μm.s^{-1})	VSL (μm.s^{-1})	VCL (μm.s^{-1})
Control	76.22 ± 4.47^{a}	18.66 ± 3.01^{a}	104.33 ± 10.46^{a}	66.11 ± 8.82^{a}	201.00 ± 9.08^{a}
NADPH 1 mM	87.67 ± 5.17^{b}	27.00 ± 2.87^{b}	123.22 ± 9.07^{b}	84.22 ± 8.15^{b}	233.44 ± 15.59^{b}
NADPH 2 mM	84.22 ± 5.67^{b}	25.00 ± 2.73^{b}	116.00 ± 5.5^{b}	78.22 ± 5.24^{b}	218.89 ± 10.56^{b}
NADPH 4 mM	66.88 ± 6.01^{c}	15.89 ± 3.48^{a}	91.78 ± 11.05^{c}	62.11 ± 6.01^{a}	187.33 ± 13.43^{c}

Values with different letters within columns significantly differ (P < 0.05).

Table 2. Effects of phenylalanine on sperm motility parameters of goat sperm samples

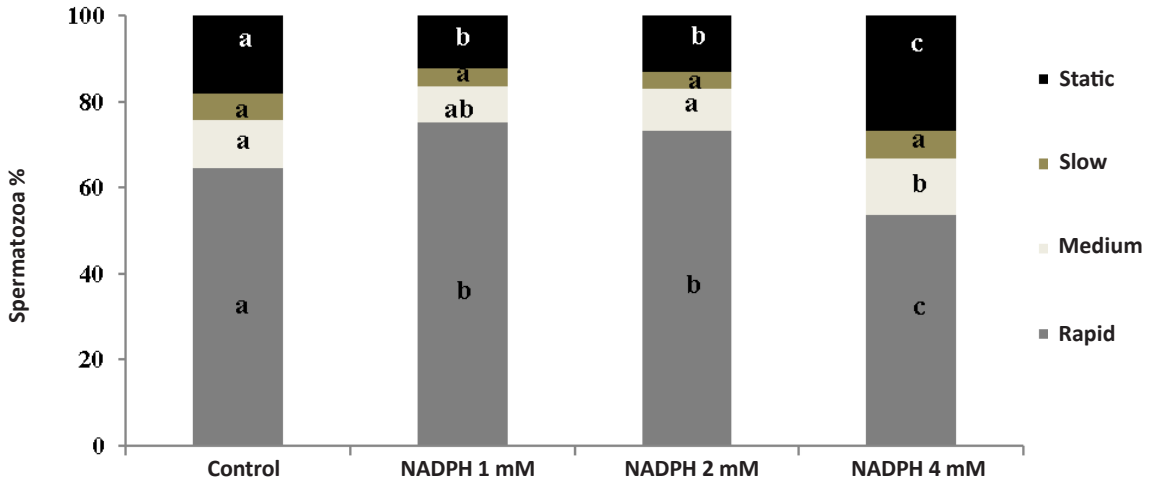
CASA parameter / Treatment	MOT (%)	PMOT (%)	VAP (μm.s^{-1})	VSL (μm.s^{-1})	VCL (μm.s^{-1})
Control	80.44 ± 2.92^{a}	19.56 ± 2.35^{a}	98.11 ± 15.92^{a}	66.22 ± 12.97^{a}	187.66 ± 10.9^{a}
Phenylalanine 1 mM	77.66 ± 5.66^{a}	17.56 ± 5.27^{a}	93.67 ± 19.12^{a}	62.22 ± 16.96^{a}	181.56 ± 16.66^{a}
Phenylalanine 2 mM	75.00 ± 3.08^{a}	16.22 ± 2.08^{a}	92.44 ± 16.83^{a}	61.11 ± 14.38^{a}	179.44 ± 12.3^{a}
Phenylalanine 4 mM	56.67 ± 6.84^{b}	10.00 ± 4.01^{b}	71.22 ± 11.5^{b}	44.56 ± 7.55^{b}	149.44 ± 20.92^{b}

Values with different letters within columns significantly differ (P < 0.05).

DISCUSSION

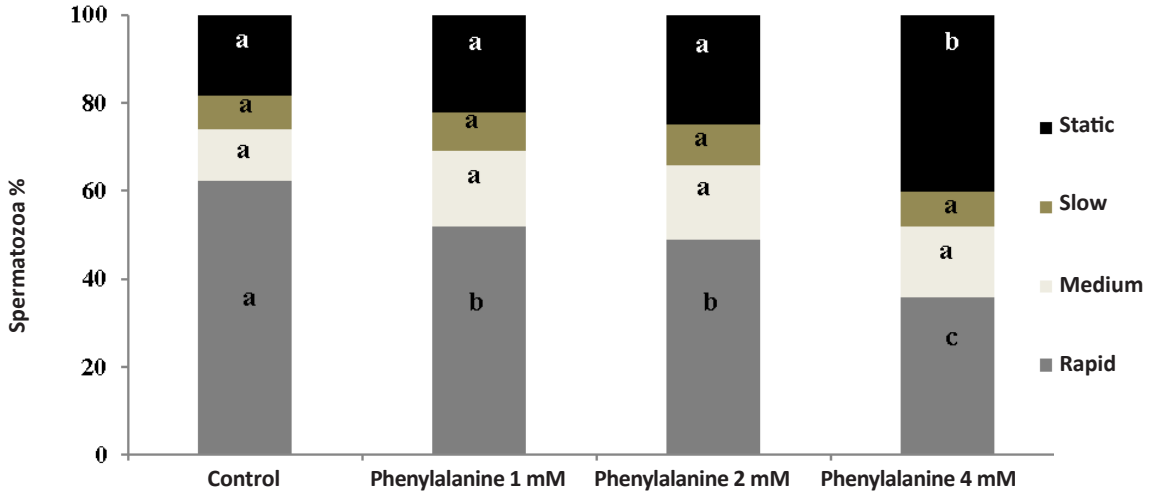
The present data shows for the first time the influences of NADPH and phenylalanine on motility parameters of goat spermatozoa. The effects of these two substrates have been a major issue of our previous studies (Alomar and Donnay, 2006; Alomar *et al*., 2016; 2018; Alomar, 2018), where we have demonstrated their abilities to induce hydrogen peroxide formation from both live and dead ram and bull spermatozoa and also their adverse effects on motility characteristics.

The principal CASA parameters showed an obvious positive effect when spermatozoa were incubated at 1 and 2 mM concentration of NADPH. In agreement with these results, 1 mM of this substrate had positively increased both PMOT % and VAP values of ram spernatozoa (Alomar *et al*., 2018). The controlled amounts of H_2O_2 produced by sperm following NADPH addition could explain these results. It must be noted that NADPH is a source of electrons for ROS generation via a proposed NADPH-oxidase reaction in spermatozoa (Aitken,



Different letters within each subpopulation category significantly differ ($P < 0.05$).

Figure 1. Effects of NADPH on the distribution of motility subpopulations of goat sperm samples



Different letters within each subpopulation category significantly differ ($P < 0.05$).

Figure 2. Effects of phenylalanine on the distribution of motility subpopulations of goat sperm samples

1997). Furthermore, NADPH appears to play a major role in sperm capacitation (Baumber *et al*., 2003; O'Flahertly *et al*., 2006). Stimulation of endogenous superoxide anion and hydrogen peroxide generation by NADPH has resulted in increased capacitation events in buffalo spermatozoa (Roy and Atreja, 2008). According to the previous authors, both O_2^- and H_2O_2 induced tyrosine phosphorylation of 95 kDa protein (p95), which is regulated by a cAMP-dependent PKA pathway. Indeed, sperm changes associated with the capacitation may include an important increase in sperm motility (Yanagimachi, 1970).

Hydrogen peroxide was generated after phenylalanine addition to bull and ram sperm showing AAAO activity in the spermatozoa of these species (Shannon and Curson, 1982b; Upreti *et al*., 1998; Alomar *et al*., 2016). It should be pointed out that the formation of H_2O_2 was reported in fresh, chilled and cryopreserved goat spermatozoa, and when phenylalanine was added to these different spermatozoa types, the H_2O_2 level was significantly raised (Alomar, 2018; 2019). In the present study, the addition of phenylalanine at either concentration reduced the values of motility parameters. In contrast, no adverse effects of phenylalanine on ram sperm motility were noted, when 1 mM of phenylalanine was added and PMOT % and VAP values were significantly raised (Alomar *et al*., 2018). In agreement with our present results, Lapointe and Sirard (1998) showed that phenylalanine had significant negative influence on bull sperms motility. These contradicted results confirm the species-specific pattern of phenylalanine effect on sperm motility, and this could be explained by different abilities of ruminants' spermatozoa to generate hydrogen peroxide formation.

Negative effects on motility values were noted when 4 mM of NADPH and phenylalanine were added. This high concentration of these oxidase substrates may be responsible for a significant increase in H_2O_2 formed by the sperm to a level causing oxidative stress. Oxidative stress is a condition associated with cellular damage and one of the most important factors contributing to the low semen quality (Bucak *et al*., 2010). Exposure of human spermatozoa to NADPH resulted in a dose-dependent generation of reactive oxygen species (ROS), which, at a critical level of intensity, induced lipid peroxidation (LPO), DNA damage and a dramatic decline in sperm motility (Twigg *et al*., 1998). An important inhibition of sperm motility after incubation with ROS was caused by a depletion of ATP and a decrease in the axonemal protein phosphorylation (De Lamirande and Gagnon, 1995).

CONCLUSION

Taken together, the data suggests that NADPH and phenylalanine have pronounced effects on goat spermatozoa motility and these effects are correlated with the concentration used. These results could be related to hydrogen peroxide generation by the spermatozoa. The addition of NADPH to semen media at the appropriate concentration can be useful as a stimulating additive for goat spermatozoa motility.

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