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# Investigating the effects of theophylline on motility, hyperactivity, and acrosome reaction of spermatozoa in rams

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## Abstract

The present study was conducted to evaluate the effect of *in vitro* treatment of ejaculated ram spermatozoa with different levels of theophylline at various incubation times on motility, hyperactivity (HA) and acrosome reaction (AR). Semen were collected from Barki rams (n=40 ejaculates) using artificial vagina twice a week. Collected semen were pooled and subjected to swim up technique in modified sperm Tyrode's albumin lactate pyruvate (S-TALP) medium supplemented with different levels of theophylline (0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml) at different incubation times (0, 1,2,3 and 4 hours). The following parameters were examined: motility, hyperactivity (HA) and acrosome reaction (AR %). The results revealed that highest motility was noticed in spermatozoa treated with 0.05 mg/ml theophylline (82.53±1.34%) immediately after dilution. While, addition of 0.01 mg/ml theophylline resulted in a significant ($P<0.05$) increase in HA% than those obtained in control (untreated) after 3 hours of incubation (5.04±0.26 *vs*.0.8±0.2). On other hand, a significant ($P<0.05$) increase in total AR% was concomitant to the increase in the concentration of theophylline with maximum achieved at 0.05 mg/ml at 3- and 4-hours post incubation (49.2±1.8; 51.2±2.5, respectively). In conclusion, under our experimental conditions, treatment of ram spermatozoa with 0.05 mg/ml of theophylline for 3-4 hours was considered the best concentration of theophylline to be used for *in vitro* induction of acrosome reaction.

***Keywords***: *Ram sperm; capacitation; hyper activation; acrosome reaction; theophylline.*

## 1. Introduction

In *vitro* fertilization (IVF) techniques are essential for the studying the basic aspects of fertilization process. The fresh ejaculated sperm are not capable for fertilization (Yanagimachi, 1994). Several sets of modifications occur in the sperm during residence in the female tract to attain the fertilizing capacity. These processes are called capacitation (Sukardi *et al.,* 1997; Bedu-Addo *et al.,* 2005; Huang, *et al.,* 2007). Different modifications of sperm cell are involved during capacitation including increase in calcium influx and lowered level of cholesterol beside lipid composition and surface protein alteration (Davis, 1981). This modification of sperm cell membrane during capacitation is a contention of membrane maturation (Sukardi *et al.,* 2001). A capacitated sperm was undergoing to be acrosome reacted without loss of fertilizing capacity (Huang *et al*., 2007). Watson and Plummer (1991) reported that, the acrosome reaction could be achieved when fresh ram spermatozoa are incubated at 39°C for four hours without using any inducing agent. in vivo the most inducers of acrosome reaction are zona pllucida and oviductal fluids (Yanagimachi, 1994). Also, it can be induced by using chemical substances such as theophylline. Theophylline is a methyl xanthine derivative that inhibits phosphodiesterase activity, thereby increasing intracellular cyclic AMP dependent processes of

sperm, including motility, capacitation, and acrosome reaction (Glogowski *et al*., 2002). The purpose of the present investigation was to evaluate the influence of different concentrations and incubation times of theophylline on the motility, hyperactivity, and acrosome reaction of ram spermatozoa.

## 2. Materials and methods

### Animal management and semen collection

Three mature Barki rams (3–5 years old with average body weight was 45 Kg) were used in the present study. The rams were kept in a farm of Animal Reproduction Research Institute (Egypt) during December 2008 to January 2019 under uniform conditions.

### Semen processing and sperm capacitation

Semen were collected from Barki rams (n=40 ejaculates) using artificial vagina twice a week. Immediately after collection, semen was kept in a water bath at (37°C) and evaluated for sperm concentration, motility, and morphology. To avoid individual variability of rams, semen was pooled. Ejaculates that contain volume of 0.5–2 ml; minimum semen concentration of 3 X 109 spermatozoa/ml; total motility higher than 80%; <10% abnormal sperm were used for the experiment according to the method described by (Chemineau et al., 1991). Split fractions (0.1ml) of the pooled semen were layered under 1 ml of sperm Tyrode's albumin lactate pyruvate (S-TALP) medium according to the method described by Younis, et al (1991) supplemented with different concentrations of theophylline (0.015, 0.02, 0.025,0.030 and 0.040 mg/ml). Semen diluted in S-TALP medium without treatment was used as a control. This technique (swim up technique) was performed in 15 ml centrifuge tubes, held at an angle of 45o and incubated in an atmosphere of 5% CO2 incubator at 39oC for 4hours. The percentage of individual motility (IM %), hyperactivity (HA %) and acrosomal status were recorded at 0, 1, 2, 3- and 4-hours post-incubation.

### Evaluation of sperm motility

A small drop of the sperm suspension (200µL) were taken from the most supernatant of swim up and examined for progressive forward motility under hot stage phase contrast microscope (40X). One hundred spermatozoa in five different field microscopes at least per slide were counted.

### Evaluation of sperm hyperactivity (HA %)

As previously reported by Kawakami et al. (1999), the hyperactivated motility percentage were determined by observing flagellar beating vigor and circular movement of sperm cells. HA % was expressed by "pluses" where (+) means HA% <20%, + means HA% 20 – 40%, ++ means HA% 40 -60%, +++ means HA% 60 – 80% and ++++ means HA% >80% (Darwish 2004).

### Evaluation of sperm acrosomal status

The acrosome reaction of spermatozoa including of incomplete and complete were recorded according to El-Amrawi and Nemetallah (1992) by using silver nitrate staining technique. In at least 100 sperm cells per slide, the percentage of acrosome reacted sperm was counted.

Three groups of the spermatozoa were identified; (1) Non acrosome reacted sperm where the outer acrosome and plasma membrane of spermatozoa were intact; (2) Incomplete acrosome reacted sperm in which, the acrosome was showing fenestrations and vesiculation with loosening between outer acrosome and plasma membrane of sperm; (3) Complete acrosome reacted sperm showing complete shed of the outer acrosome membrane with cup-shaped appearance. Total acrosomes were equal to incomplete plus complete acrosome percentages.

## 3. Statistical analysis

Data were expressed as mean ± SEM that is obtained from 10 replicates for motility, hyperactivity and acrosome reaction and were analyzed using Costat computer program; version 3.03, Copyright (1986) Cottort Software. Data from 10 replicates were subjected to analysis of variance (two-way ANOVA) to clarify the effect of theophyline concentrations and incubation times. P-values less than 0.05 were considered statistically significant.

## 4. Results

### Effect of different concentrations of theophylline on ram sperm functions

### Effect on sperm motility (IM)

Maximum increase of IM was noticed in spermatozoa treated with 0.05 mg/ml theophylline (82.53±1.34%) immediately after dilution. However, there was no significant difference between different concentrations of theophylline and control at different incubation periods. Incubation of ram spermatozoa at 37oC for 3 hours resulted in a significant (P<0.05) decrease in the individual motility of ram spermatozoa as shown in Table 1.

**Table (1): Effect of different theophylline concentrations (mg/ml) and incubation time on motility of ram spermatozoa (Means ±SE)**

| Concentration | Incubation time (hours) | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 |
| Control | 74.0±1.2$^{Ba}$ | 75.0±1.6 $^{Aa}$ | 69.0±3.8 $^{Aab}$ | 62.0±5.0 $^{Ab}$ | 47.0±3.8 $^{Ac}$ |
| 0.015 | 78.0±1.6$^{ABa}$ | 79.5±1.1 $^{Aa}$ | 78.0±1.3 $^{Aa}$ | 65.0±5.0 $^{Ab}$ | 48.0±4.0 $^{Ac}$ |
| 0.020 | 77.5±0.8$^{ABab}$ | 81.5±0.7 $^{Aa}$ | 71.5±2.7 $^{Aab}$ | 64.5±5.1 $^{Ab}$ | 47.5±6.6 $^{Ac}$ |
| 0.025 | 78.0±1.3$^{ABa}$ | 80.5±1.5 $^{Aa}$ | 73.5±3.0 $^{Aab}$ | 63.0±5.0 $^{Ab}$ | 47.5±5.7 $^{Ac}$ |
| 0.030 | 80.5±1.7$^{Aa}$ | 78.0±1.8 $^{Aa}$ | 77.5±1.7 $^{Aa}$ | 69.5±2.7 $^{Aa}$ | 52.5±6.4 $^{Ab}$ |
| 0.040 | 79.0±2.0$^{ABa}$ | 73.0±3.2 $^{Aa}$ | 68.5±3.6 $^{Aa}$ | 63.5±5.0 $^{Aa}$ | 49.0±7.3 $^{Ab}$ |
| 0.050 | 82.5±1.3 $^{Aa}$ | 75.0±2.5 $^{Aa}$ | 66.0±6.2 $^{Aab}$ | 51.0±8.0$^{Abc}$ | 46.0±7.0 $^{Ac}$ |

Means with different alphabetical superscripts in the same columns A, B…. and different rows a,b…. are significant at least at $p < 0.05$.

### Effect on sperm hyperactivity (HA)

Table 2 describes the influence of different theophylline concentrations and increasing incubation time on HA%. Maximum increase of HA% was noticed in spermatozoa treated with 0.015

mg/ml theophylline (5.04±0.26). HA% undergo highly significant (P<0.01) improvement after 2, 3 and 4 hours of incubation.

**Table (2): Effect of different theophylline concentrations (mg/ml) and incubation time on hyperactivity of ram spermatozoa (Means ±SE)**

| Concentration | Incubation time (hours) | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 |
| Control | 0.0±0.0 Ac | 0.0±0.0 Dc | 0.4±0.2 Bbc | 0.8±0.2Cb | 1.6±0.1 Aa |
| 0.015 | 0.0±0.0 Ad | 1.8±0.1BCc | 3.8±0.6Ab | 5.4±0.2 Aa | 4.0±0.7Ab |
| 0.020 | 0.0±0.0 Ac | 2.0±0.0BCb | 3.2±0.1Aa | 4.2±0.1 ABa | 3.8±0.7 Aa |
| 0.025 | 0.0±0.0 Ac | 2.4±0.1 ABCb | 4.0±0.2Aa | 4.6±0.2ABa | 4.2±0.7Aa |
| 0.030 | 0.0±0.0Ab | 1.4±0.2Cb | 3.6±0.8Aa | 4.0±0.7ABa | 4.2±0.7Aa |
| 0.040 | 0.0±0.0 Ab | 2.6±0.6ABa | 3.6±0.6 Aa | 4.0±0.6ABa | 3.6±0.6 Aa |
| 0.050 | 0.0±0.0 Ab | 3.2±0.1Aa | 3.6±0.7 Aa | 2.8±0.8 Ba | 1.6±0.7Aab |

Means with different alphabetical superscripts in the same columns A, B.... and different rows a,b.... are significant at least at $p < 0.05$.

## Effect on sperm incomplete acrosome reaction (AR %)

Treatment of spermatozoa with 0.05 mg/ml theophylline resulted in a significant (P<0.05) increase in IAR% immediately after dilution and for 2 hr. of incubation. While, a highly significant (P<0.01) increase in IAR% was observed with 0.03 mg/ml after 3 hours of incubation period, then it was significantly (P<0.05) declined as the incubation period increased (Table 3).

**Table (3): Effect of different theophylline concentrations (mg/ml) and incubation time on incomplete acrosome reaction of ram spermatozoa (Means ±SE)**

| Concentration | Incubation time (hours) | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 |
| Control | 0.0±0.0 Bc | 0.0±0.0 Dc | 0.0±0.0 Dc | 11.00±1.1 Db | 21.3±1.6 BCa |
| 0.015 | 0.0±0.0 Bd | 9.5±0.6 BCc | 27±0.9 Ba | 27.30±1.3 Ca | 22.7±2.0 Ab |
| 0.020 | 0.0±0.0 Bd | 7.2±1.0 Cc | 20.5±1.3 Cb | 27.50±1.3 Ca | 19.3±1.3 Cb |
| 0.025 | 0.0±0.0 Be | 11.1±1.2 BCd | 30.1±1.4 Bb | 36.10±1.5 Ba | 23.3±1.4 BCc |
| 0.030 | 0.0±0.0 Bd | 6.5±0.9 Cc | 26.2±1.4 Bb | 41.30±2.3Aa | 26.8±1.1 Bb |
| 0.040 | 0.0±0.0 Be | 13.6±1.3 Bd | 31.3±1.3 Bb | 35.50±2.0 Ba | 25.6±1.6Bc |
| 0.050 | 8.2±0.8 Ad | 35.5±2.1 Ab | 43.5±2.8Aa | 34.40±1.8 Bb | 20.8±1.5 BCc |

Means with different alphabetical superscripts in the same columns A, B.... and different rows a,b.... are significant at least at $p < 0.05$.

## Effect on complete acrosome reaction (AR %)

It was clear that high concentrations of theophylline (0.05 mg/ml) significantly (P<0.05) increased the CAR% as compared to untreated spermatozoa immediately after dilution and all over the incubation period as shown in Table 4. Moreover, the maximum CAR% was achieved after 4 hours of incubation period at 37oC in semen samples treated with 0.03 and 0.05 mg/ml of theophylline.

**Table (4): Effect of different theophylline concentrations (mg/ml) and incubation time on complete acrosome reaction of ram spermatozoa (Means ±SE)**

| Concentration | Incubation time (hours) | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 |
| Control | 0.0±0.0Bc | 0.0±0.0 Dc | 0.0±0.0 Fc | 2.7±0.5Eb | 11.2±0.9 Ea |
| 0.015 | 0.0±0.0 Bd | 5.3±1.2 Cc | 14.1±1.2 Cb | 20.7±1.9Ca | 21.2±1.4 Da |
| 0.020 | 0.0±0.0 Bd | 4.1±0.7 Cc | 5.7±0.8 Ec | 14.5±0.6 Db | 29.9±2.2Ca |
| 0.025 | 0.0±0.0Bd | 9.3±0.7 Bc | 10.1±0.8 Dc | 21.0±0.94 Cb | 45.3±1.4Ba |
| 0.030 | 0.0±0.0 Be | 5.7±0.8 Cd | 14.1±1.2 Cc | 24.6±1.5 Cb | 55.8±2.8Aa |
| 0.040 | 0.0±0.0 Bd | 15.7±1.4 Ac | 33.5±1.2 Ab | 37.5±1.5 Bb | 42.3±2.0 Ba |
| 0.050 | 1.5±0.3 Ad | 17.1±0.7 Ac | 26.2±1.4 Bb | 49.2±1.8 Aa | 51.2±2.5 Aa |

Means with different alphabetical superscripts in the same columns A, B.... and different rows a,b.... are significant at least at $p < 0.05$.

## 5. Discussion

In the present study, the effect of theophylline on ram sperm functions was studied. The present study revealed that maximum increase of IM was noticed in spermatozoa treated with 0.05 mg/ml theophylline (82.53±1.34%) immediately after dilution. In agreement to our data, Lindemann (1983) reported that treatment of bull spermatozoa with theophylline resulted in increased motility due to the marked increase in intracellular cAMP. In this respect, Bhatnagar & Anand (1982) stated that theophylline inhibited cyclic nucleotide phosphodiestrase activity in midpiece and tail of buffalo spermatozoa which might serve as a physiological regulation of sperm motility. In contrast, Darwish (2004) and Marie (2005) reported that, low concentration (up to 10 mg/ml) of theophylline was found to increase sperm motility and HA%. However, when theophylline concentration exceeded 10 mg/ml, motility was highly (P<0.01) decreased. Maximum motility was achieved after 2 hours of incubation. The difference in methodology and samples used has probably resulted in the disparate results obtained. Elaheh Gorji et al., (2018) reported that, addition of theophylline will lead to improvement in progressive motility. On other hand, Yoshioka et al. (2003) reported that a significantly ($P < 0.05$) increase in fertilization rate was achieved in the presence of theophylline. In addition, Longhlin et al. (1992) showed that addition of theophylline (20 mM) significantly increased the penetration rate of sperms. Moreover, Sinha et al., (1995) reported that, the viability of post-thaw motility of buck spermatozoa was increased from 45% to 52% in the presence of theophylline before freezing.

The current study revealed a highly significant (P<0.05) increase in incomplete acrosome reacted spermatozoa using 0.05 mg/ml after 2 hours of incubation. Furthermore, spermatozoa treated with 0.05 mg/ml had higher percentage of complete and

AR when incubation time was prolonged to 4 hours. The total AR was also maximal at this concentration. However, Darwish (2004) and Marie (2005) obtained similar results using 10 mg/ml theophylline. They considered 10 mg/ml was the best concentration of theophylline to be used in buffalo IVF programs. This difference may be attributed to species differences. The present study detected a highly significant ($P<0.0001$) effect of theophylline, incubation time and interaction between them on complete AR% of ram spermatozoa. In this respect, Takahashi and First (1993) observed that in vitro fertilization of bovine oocytes after preincubation of bovine spermatozoa with theophylline (2.5-5 mM) for 4-5 hours resulted in increased fertilization rate and achieved 18% blastocyst rate. In addition, El-Gaafary et al. (1993) found that theophylline treated bull spermatozoa were able to penetrate cow cervical mucous and achieved a significant ($P<0.01$) increase in penetration rate (57.10%) in zona-free hamster oocyte.

In conclusion, treatment of ram spermatozoa with 0.05 mg/ml of theophylline for 3-4 hours was considered the best level of theophylline to be used for in vitro induction of acrosome reaction.

**Conflicts of interests:** The author declares that he has no competing interests.

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