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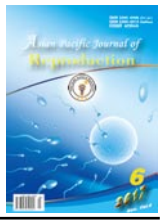

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Effects of penicillamine, hypotaurine, and epinephrine on motility, hyperactivity, acrosome reaction of fresh ram sperm

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ABSTRACT

Objective: To determine the effects of different concentrations of penicillamine, hypotaurine, and epinephrine (PHE) as well as incubation time on motility, hyperactivity and acrosome reaction (AR) of ram sperm *in vitro*. **Methods:** Freshly ejaculated spermatozoa from three ram were collected, pooled and subjected to swim up technique in modified sperm Tyrode's albumin lactate pyruvate medium supplemented with different concentrations of PHE (10, 20, 30, 40, 50, 75 and 100 mM/mL). Then best concentrations were compared and examined for motility, hyperactivity and AR. **Results:** A high concentrations of PHE (30, 40, 50, 75 and 100mM/mL) showed a significant increase in motility when compared to control immediately after dilution and exist for the first and second hour of incubation period. However, when longer incubation time were used, a significant (P<0.05) decrease in motility was achieved. Similar finding was observed in hyperactivity. Stained semen samples showed a maximum percentage of incomplete AR after 1 h incubation corresponding to 50 and 75 mM/mL of PHE; however, spermatozoa treated with 75 mM/mL had a higher tendency to undergo complete AR after further incubation up to 4 h. A dose dependent relationship was detected where the maximum value of total AR was shown in spermatozoa treated with 75 mM/mL PHE for 4 h. **Conclusions:** To obtain better motility, hyperactivity and AR, treatment of ram spermatozoa with 75 mM/mL PHE for 4 h before being used in insemination was considered the best concentration of PHE to be used in the process of *in-vitro* fertilization.

1. Introduction

Successful fertilization depends upon several interrelated physiological processes all of which must take place in a coordinated manner. One such process is sperm capacitation and the acrosome response. Capacitation is a key step through which sperm become fertilization-competent and hence is essential for the success and development of assisted reproductive[1]. Capacitation include layer alterations, incorporating changes in lipid arrangement, surface properties, smoothness, influx of calcium ions, increase in cyclic adenosine monophosphate levels and changes in some enzyme activities, such as protein tyrosine kinase[2]. These biochemical alterations enable the sperm to undergo the culminating point, which is the acrosome reaction (AR). There are various chemical agents that might be utilized both to fortify and keep up sperm motility, which is a mixture of penicillamine, hypotaurine and

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epinephrine (PHE), the most as often as possible utilized substance to invigorate sperm motility[3-5]. Penicillamine has been appeared to increase the proportion of sperm that undergo to AR, when utilized in a mixtuer of epinephrine[6]. A β -amino corrosive (taurine or hypotaurine) keeps up sperm motility, viability and oocyte entrance yet may likewise assume a part in sperm capacitation. Epinephrine has been appeared to actuate of sperm motility[7]. While the mix of epinephrine and hypotaurine increase the oocyte penetration rate by bovine spermatozoa and subsequent pronuclei formation[8]. Thus, the present study aimed to determine the effects of different concentrations of PHE and incubation time on motility, hyperactivity (HA) and AR of ram sperm *in vitro*.

2. Materials and methods

2.1. Preparation of PHE

All the chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

The PHE supplement was prepared by first making stock solutions of 2 mM penicillamine and 1 mM hypotaurine in saline. An epinephrine stock solution was made by adding 165 mg of 60% sodium lactate and 50.00 mg sodium meta-bisulfate to 50 mL of 18 megohm water. After adjusting the pH to 4, 1.83 mg of epinephrine was then added to 40 mL of this solution. The PHE stock solution is prepared by adding 5 mL of the penicillamine stock, 5 mL hypotaurine stock, and 4 mL of epinephrine stock to 6 mL physiological saline. The PHE stock solution was placed into vials, wrapped in aluminum foil and stored at -20 ℃. On the day of use, 40 μL of the PHE stock solution was added to each 960 μL of Sperm-TL medium. Final concentration of the medium consisted of 20 μM penicillamine, 10 μM hypotaurine, 2 μM epinephrine, 21 μM sodium meta-bisulfite and 118 μM sodium lactate.

2.2. Semen processing and sperm capacitation

2.2.1. Semen collection

Semen were collected from 3 ram aged 2-3 years (n=40, ejaculation) using an artificial vagina twice per week. After collection of semen, the samples were immediately brought to the laboratory and placed in a water bath at 37-38 ℃. In each ejaculate, volume, concentration, sperm motility and morphology were assessed within 30 min after semen collection by using a light microscope. Samples with motility higher than 60%; <10% abnormal sperm were pooled together to avoid individual variation of rams according to Chemineau P *et al*[9].

2.2.2. Sperm capacitation

Split fractions (0.1 mL) of the pooled semen were layered under 1 mL of S-TALP medium according the method described by Younis AI *et al*[10], and supplemented with different concentrations of PHE (10, 20, 30, 40, 50, 75 and 100 mM/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 a 45 angle and incubated in 5% CO_2 incubator at 39 ℃. Individual motility percentage (IM%), hyperactivity motility percentage (HA%) and acrosome status were recorded at 0, 60, 120, 180 and 240 min of post-inoculation.

2.2.3. Evaluation of sperm individual motility

Percentage of progressive forward motility was subjectively estimated in a small drop (200 μL) of the sperm suspension from the most supernatant of swim up and covered by a cover slip, and examined under phase contrast microscope (40 ×) equipped with a heated plate (37 ℃). Only progressive forward motility was considered among different treatments and control. At least 100 spermatozoa in five different fields were counted.

2.2.4. Evaluation of sperm hyperactivity

HA% was determined by recording the percentage of sperm cells with flagellar beating vigor and circular movement. HA% was considered from IM% and expressed by 'pluses' where (+) means HA% <20%, + means HA% 20%-40%, ++ means HA% 40%-60%, +++ means HA% 60%-80% and ++++ means HA%>80%[11].

2.2.5. Evaluation of sperm acrosomal status

Percentages of incomplete and complete acrosome reacted spermatozoa were determined by silver nitrate staining technique according to El-Amrawi GA and Nemetallah BR[12]. The percentage of acrosome reacted sperm was counted in at least 100 sperm cells per slide. The spermatozoa were classified into three groups: (1) spermatozoa with an intact plasma and outer acrosomal membrane; (2) Spermatozoa with incomplete AR show fenestrations, vesiculation and loosening between plasma membrane and outer acrosomal membrane; (3) Spermatozoa with complete AR showing complete loss of the outer acrosomal membrane leaving cup-shaped appearance. Both incomplete and complete AR percentages were considered collectively as total AR.

2.3. Evaluation of fertilizing capacity of treated ram spermatozoa

2.3.1. Oocytes recovery and selection

Ovaries of slaughtered ewes of unknown reproductive history were collected from local abattoir within 20 min post-slaughter, and were transported in thermo box containing a pre-warmed sterile saline supplemented with antibiotic (100 μg/mL streptomycin and 100 IU/mL penicillin) at 37 ℃. The average transport time of the ovaries to the laboratory was 2 h. The ovaries were then washed with warm sterile saline to remove adhering blood. Oocytes were harvested from the ovaries by aspiration technique. Follicular fluid from all visible

surface follicles ranged from 2-8 mm in diameter were aspirated using 18 G needle fitted to a 5 mL disposable syringe containing modified phosphate buffer saline supplemented with 10% fetal calf serum (FCS, Sigma, USA) enriched with sodium pyruvate (0.036 g/mL), and the above mentioned antibiotics. The contents were pooled in a sterile 15 mL centrifuge tubes and allowed to settle for 5 min in laminar flow hood. The clear fluid was then discarded while the bottom portion was poured in Petri dish containing hormone free Tissue Culture medium-199 (TCM-199, HEPES modification with Earle's salt and L-glutamine, Sigma, USA) media examined under stereomicroscope under low magnification (10 ×-20 ×) for the presence of oocytes. All the aspirated cumulus-oocyte complexes (COCs) with homogenous cytoplasm were used in the study. The oocytes were washed three times in TCM-199 plus 10% FCS and 1% antibiotic -antimycotic (Gibco, Switzerland) according to Datta TK *et al*[13].

2.3.2. *In vitro oocytes maturation and fertilization*

COCs were cultured in 50 μL of TCM-199+10% FCS and 1% antibiotic-antimycotic covered with equilibrated sterile Millipore filtered (0.45 μm) light-weight paraffin oil (Sigma, USA) in four-well culture plate (10 to 15 oocytes per droplet) for 24 h in a CO_2 incubator (5% CO_2 and 95% relative humidity) at 39 ℃. Then the oocytes were assessed for maturation signs. The oocytes showing expanded cumulus cells, perivitelline space or extruded first polar body were selected for *in-vivo* fertilization (IVF)[14]. Selected *in vitro* matured COCs were washed three times in S-TALP medium. The surrounding cumulus cells were partially removed by gentle pipetting. Five to ten oocytes were then allocated to each minidrop, containing F-TALP medium, in polystyrene culture dish covered with warm sterile paraffin oil and incubated for 1 h until spermatozoa were added. Pre-treated semen with best concentration of PHE (determined on the bases of the best motility, hyperactivity, percentage of incomplete AR with high tendency to undergo complete AR, in addition to total AR) was used for insemination. Sperm cell concentration of semen was estimated after swim up and a total of 2 106 sperm cell were added to the mini drops so that the final volume of each mini drop is 50 μL. Oocytes and spermatozoa were incubated together for 18-24 at 39 ℃ under 5% CO_2 in air and at maximum humidity. At the end of the incubation period, oocytes/zygotes were examined for the evidence of fertilization. Oocytes with male and female pronuclei ± secondary polar bodies were considered to be normally fertilized. Oocytes with more than one sperm head or male pronuclei were considered to be polyspermic. The fertilization rate and penetration rates were recorded[15].

2.4. Statistical analysis

The obtained data were analyzed statistically using costat computer program version 3.03, Copyright (1986) Cottort Software. Data were subjected to analysis of variance (two way ANOVA, Duncan's Multiple Range Test) to clarify the effect of different capacitating agents concentrations and incubation time. On the other hand, in vitro penetration and fertilization rates were analysed by using *chi*-square analysis (χ^2). Data are expressed as means±SE. $P<0.05$ was considered significant.

Table 1

Effect of different PHE concentrations and incubation time on motility of ram spermatozoa (means±SE).

Treatments (mM/mL)	Incubation time (h)				
	0	1	2	3	4
Control	76.50±1.30^{Ba}	73.00±0.81^{Ba}	66.00±2.66^{Bb}	59.50±2.9^{3Ab}	46.50±3.42^{Ac}
10	79.00±1.24^{ABa}	79.00±1.94^{Aa}	76.00±2.56^{Aa}	61.00±4.33^{Ab}	38.50±3.94^{Ac}
20	80.50±1.38^{Aba}	77.00±1.69^{Aba}	78.50±1.83^{Aa}	62.50±4.42^{Ab}	44.50±5.55^{Ac}
30	82.50±0.83^{Aa}	80.50±1.38^{Aa}	79.50±1.16^{Aa}	66.00±2.86^{Ab}	42.00±3.26^{Ac}
40	82.50±0.83^{Aa}	78.00±1.69^{Aba}	79.00±1.63^{Aa}	61.50±3.42^{Ab}	37.50±2.50^{Ac}
50	81.50±0.83^{Aba}	80.00±1.49^{Aa}	77.00±2.13^{Aa}	70.00±4.28^{Aa}	47.50±4.03^{Ab}
75	82.50±0.83^{Aa}	81.00±1.40^{Aa}	78.00±1.69^{Aa}	66.50±4.15^{Ab}	42.00±4.54^{Ac}
100	82.00±1.33^{Aa}	82.00±1.33^{Aa}	77.00±2.00^{Aa}	67.50±2.91^{Ab}	44.50±4.62^{Ac}

Means with different alphabetical superscripts in the same columns A, B, C, D and different rows a,b,c are significantly different at $P<0.05$.

Table 2

Effects of different PHE concentrations and incubation time on hyperactivity of ram spermatozoa.

Treatements (mM/mL)	Incubation time (h)				
	0	1	2	3	4
Control	0.00±0.00^{Ab}	0.00±0.00^{Bb}	0.20±0.13^{Bb}	1.20±0.48^{Ba}	1.60±0.16^{Ca}
10	0.00±0.00^{Ad}	2.40±0.33^{Ac}	3.60±0.44^{Ab}	4.60±0.26^{Aa}	2.40±0.26^{ABCc}
20	0.00±0.00^{Ac}	2.80±0.48^{Ab}	4.80±0.53^{Aa}	5.00±0.29^{Aa}	2.80±0.24^{ABb}
30	0.00±0.00^{Ac}	3.40±0.49^{Aab}	4.80±0.71^{Aa}	4.20±0.32^{Aa}	2.60±0.26^{ABb}
40	0.00±0.00^{Ac}	2.80±0.61^{Ab}	4.00±0.36^{Aa}	3.80±0.24^{Aa}	2.20±0.13^{ABCb}
50	0.00±0.00^{Ab}	3.00±0.51^{Aa}	4.00±0.29^{Aa}	4.40±0.61^{Aa}	3.20±0.48^{Aa}
75	0.00±0.00^{Ac}	4.20±0.48^{Aa}	4.60±0.26^{Aa}	4.60±0.57^{Aa}	2.80±0.13^{ABb}
100	0.00±0.00^{Ad}	3.80±0.64^{Ab}	5.20±0.38^{Aa}	3.40±0.16^{Ab}	2.00±0.00^{BCc}

Means with different alphabetical superscripts in the same columns A, B, C, D and different rows a,b,c are significantly different at $P<0.05$.

3. Results

Effects of different PHE concentrations and different incubation time on motility of ram spermatozoa were presented.

3.1. Effect on IM%

Data regarding the effects of different PHE concentrations and different incubation time on motility of ram spermatozoa were presented in Table 1. Irrespective of incubation time, there was a highly significant increase (P<0.05) in motility percentage of spermatozoa treated with high concentrations of PHE (30, 40, 50, 75 and 100 mM/mL) as compared to untreated spermatozoa (control). These improvements in IM were observed immediately after dilution and exist for the first and second hour of incubation period. During the last two hours of incubation period, there were no any significant difference in IM between control and PHE treated samples. With respect to time factor, there was a non-significant decrease in IM after 1st two hours; then a significant decrease (P<0.05) simultaneous with incubation time in all treated and control semen samples.

3.2. Effect on HA%

It was demonstrated that a significant increase in HA% for different concentrations of PHE as compared to control one (Table 2). Irrespective to concentration, maximum HA% was detected in spermatozoa after 2-hour incubation.

3.3. Effect on incomplete AR percentage (IAR%)

Maximum IAR% was noticed at 75 mM/mL PHE concentration compared to other treated and untreated spermatozoa at 1st, 2nd, and 3rd hour of incubation (Table 3). A dose response relationship

Table 3
Effect of different PHE concentrations and incubation time on incomplete acrosome reaction of ram spermatozoa (Means ±SE)

Treatments (mM/mL)	Incubation time (h)				
	0	1	2	3	4
Control	0.00±0.00^{Ac}	0.00±0.00^{Ec}	0.00±0.00^{Cc}	16.10±2.13^{Cb}	22.30±2.15^{Ba}
10	0.00±0.00^{Ad}	6.80±1.11^{Dc}	24.90±1.29^{Bb}	32.20±1.44^{Ba}	32.30±2.71^{Aa}
20	0.00±0.00^{Ad}	5.10±0.79^{Dc}	26.80±1.57^{Bb}	37.20±3.30^{Ba}	25.10±1.25^{Bb}
30	0.00±0.00^{Ad}	0.00±0.00^{Ec}	21.90±1.55^{Bb}	34.10±2.21^{Ba}	34.20±1.49^{Aa}
40	0.00±0.00^{Ad}	20.60±1.39^{Bc}	27.50±1.87^{Bab}	31.20±1.54^{Ba}	25.00±1.31^{Bb}
50	0.00±0.00^{Ac}	15.20±1.48^{Cc}	26.50±2.80^{Bb}	38.20±1.70^{Ba}	33.99±2.03^{Aa}
75	0.00±0.00^{Ac}	27.50±1.69^{Ab}	50.10±2.41^{Aa}	46.70±3.39^{Aa}	23.70±1.28^{Bb}
100	0.00±0.00^{Ad}	16.90±1.31^{Cc}	25.50±1.39^{Bb}	33.20±1.97^{Ba}	27.90±2.00^{Bb}

Means with different alphabetical superscripts in the same columns A, B, C, D and different rows a,b,c are significantly different at P<0.05.

Table 4
Effect of different PHE concentrations and incubation time on complete acrosome reaction of ram spermatozoa.

Treatments (mM/mL)	Incubation time (h)				
	0	1	2	3	4
Control	0.00±0.00^{Ac}	0.00±0.00^{Bc}	0.00±0.00^{Cb}	5.30±0.73^{Ca}	17.70±1.16^{Ca}
10	0.00±0.00^{Ac}	1.50±0.39^{Bc}	12.10±0.92^{Cc}	31.90±2.06^{Cb}	34.50±2.99^{Ba}
20	0.00±0.00^{Ac}	3.20±0.87^{Ac}	27.30±1.77^{Bb}	30.20±1.76^{Bb}	43.50±2.03^{Ba}
30	0.00±0.00^{Ac}	0.00±0.00^{Dc}	24.60±1.71^{Bb}	34.50±1.32^{Bb}	44.20±2.18^{Ba}
40	0.00±0.00^{Ad}	3.50±0.93^{Ad}	27.80±2.25^{Ac}	35.10±1.34^{Ab}	35.60±1.20^{Aa}
50	0.00±0.00^{Ac}	17.50±0.95^{Bc}	31.50±1.53^{Cb}	44.60±1.22^{Ca}	52.40±1.63^{Ca}
75	0.00±0.00^{Ac}	17.70±1.46^{Bc}	32.00±1.83^{Cc}	43.90±1.55^{Cb}	49.10±2.66^{Ba}
100	0.00±0.00^{Ac}	8.50±0.95^{Ac}	17.80±1.47^{Bb}	30.40±1.49^{Bb}	45.90±1.87^{Ba}

Means with different alphabetical superscripts in the same columns A, B, C, D and different rows a,b,c are significantly different at P<0.05.

Table 5
Effect of different PHE concentrations and incubation time on total acrosome reaction of ram spermatozoa.

Treatments (mM/mL)	Incubation time (h)				
	0	1	2	3	4
Control	0.00±0.00^{Ac}	0.00±0.00^{Ec}	0.00±0.00^{Ec}	21.40±2.45^{Cb}	40.00±2.83^{Da}
10	0.00±0.00^{Ae}	8.30±1.35^{Dd}	37.00±1.48^{Dc}	64.10±2.32^{Bb}	66.80±5.70^{Ba}
20	0.00±0.00^{Ad}	8.30±1.51^{Dc}	54.10±2.06^{Bb}	67.40±2.49^{Ba}	68.60±2.81^{BCa}
30	0.00±0.00^{Ad}	15.20±1.48^{Bc}	51.10±4.51^{Bb}	72.70±3.11^{Aa}	78.10±4.21^{Aa}
40	0.00±0.00^{Ae}	24.10±1.20^{Cd}	55.30±2.67^{Bc}	66.30±2.07^{Ba}	60.60±0.89^{Cb}
50	0.00±0.00^{Ad}	17.50±0.95^{Ed}	53.40±3.08^{Cc}	78.70±3.43^{Bb}	86.60±3.12^{ABa}
75	0.00±0.00^{Ad}	45.20±2.92^{Ac}	82.10±3.77^{Aa}	90.60±4.66^{Aa}	72.80±2.58^{Bb}
100	0.00±0.00^{Ae}	25.40±1.23^{Cd}	43.30±2.30^{CDc}	63.60±1.73^{Bb}	73.80±1.92^{Ba}

Means with different alphabetical superscripts in the same columns A, B, C, D and different rows a,b,c are significantly different at P<0.05.

in IAR% was noticed for heparin concentration up to 75 mM/mL followed by relative decrease in response to higher concentrations.

3.4. Effect on complete AR percentage (CAR%)

A significant increase (P<0.05) in CAR% was associated to different PHE treatments (Table 4). However, 50 and 75 mM/mL concentrations achieved the significantly highest effects from the first to last hour of incubation. On the other hand, incubation for 4 h resulted in a highly significant increase (P<0.05) in CAR% as compared to other incubation periods.

3.5. Effect on total AR percentage (TAR%)

A significant increase (P<0.05) of TAR% was noticed in all PHE treated spermatozoa (Table 5). Maximum TAR% was achieved after treatment of ram spermatozoa with 75 mM/mL PHE as compared to others. A significant increase in TAR% was concomitant to the increase in incubation time for 3-4 h.

3.6. Effect on in vitro fertilization

A significant increase (P<0.05) in the penetration and fertilization rates of ovine oocytes by sperm previously capacitated with 75 mM/mL PHE as compared to control (Table 6).

Table 6
Effect of best concentration of different capacitating active compounds on in vitro fertilization of ovine oocytes.

Treatments	Number of matured oocytes	Penetration Rate (%)	Fertilization Rate (%)	Abnormal Fertilization (%)
Control	90	40/90(44.40)^{b}	8/35(22.86)^{b}	7/35(20.00)^{b}
PHE	26	14/26(53.85)^{a}	13/26(50.00)^{a}	1/26(3.85)^{a}

Means with different alphabetical superscripts in the same columns a,b,c are significantly different at P<0.05.

4. Discussion

As a matter of fact, high sperm motility is required to accomplish fertilization. Moreover, hyperactivated motility was found to be very important to enable the spermatozoa to penetrate the zona pellucid[16,17], and it was considered as a biomarker of the ability of spermatozoa to undergo capacitation. Furthermore, Gadella BM *et al*[18] reported that the ability of the capacitated spermatozoa to undergo AR is a prerequisite for the success of the process of IVF. Consequently, the present study was concerned with the ability of PHE to improve sperm motility, to enhance hyperactivity, and to stimulate spermatozoa to undergo incomplete AR with high tendency to detach acrosome to form CAR after further incubation. In the present study, different concentrations of PHE were used. Regarding the effect of PHE on motility, treatment of ram spermatozoa with high concentrations of PHE (30, 40, 50, 70 and 100 mM/mL) resulted in improvement of IM% and HA% following 2 h of incubation as compared to control group. In the present findings, spermatozoa treated with 50 and 75 mM/mL of PHE showed maximum increase in the IAR% after 1 h incubation. In addition, spermatozoa treated with 75 mM/mL PHE tended to show that CAR after longer incubation time was very high if compared to other concentrations. Moreover, spermatozoa treated with 75 mM/mL had a higher tendency to undergo CAR after further incubation up to 4 h, and the TAR was significantly increased. In order to obtain better motility, hyperactivity and AR, treatment of ram spermatozoa with 75 mM/mL PHE for 4 h before being used in insemination was considered the best concentration of PHE to be used in the process of IVF. Similar effect was observed in ram[19] and bull[20]. This increase in motility and HA% is due to the inhibiting ability of hypotaurine in reactive oxygen species and activate sperm motility. On other hand, epinephrine as a kind of catecholamine might stimulate soluble adenyl cyclase and result in increase of cyclic adenosine monophosphate in the cytoplasm of sperm[21]. It might speed the flagellar beat of mice sperm by a non-receptor-mediated mechanism[22]. Moreover, addition of D-penicillamine to culture medium increased the viability of bull sperm[23]. It was observed that Long periods of incubation of boar semen may also increase damage to DNA of spermatozoa due to the generation of reactive oxygen species[24]. Treatment of bull semen with heparin and PHE for a shorter period of time (4 h) had no effect on the DNA of spermatozoa[25], which supported the present findings where we used 4 h as maximum incubation period. Increasing the fertilizing ability of ram spermatozoa is the target of capacitation process, thus treated with the best concentration of 75 mM/mL PHE to increase the ability of ram spermatozoa to penetrate and fertilize matured ovine oocytes *in vitro*. A significant effect was noticed in penetration rate and fertilization rate of oocytes inseminated with spermatozoa treated with 75 mM/mL PHE as compared to control one (P<0.05). It was previously demonstrated that PHE resulted in an increase of 23% in the cleavage rate[5]. Similar finding was obtained in this study. In addition, it was reported that the addition of PHE to IVF medium improved motility and sperm parameters associated with bull fertility examined by computer-assisted sperm analysis[26]. Similar finding was obtained by Gonçalves FS *et al*[25]. Furthermore, it was concluded that the combination of the PHE mixture (20 μM D-penicillamine, 10 μM hypotaurine and 1 μM epinephrine) and theophylline added to IVF medium could synergistically speed sperm motility and penetration of bovine oocytes[20]. They can achieve stable normal fertilization and blastocyst development in any bull.

In conclusion, to obtain better motility, hyperactivity and AR, treatment of ram spermatozoa with 75 mM/mL PHE for 4 h before being used in insemination was considered the best concentration of PHE to be used in the process of IVF.

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