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Effect of Ca^{2+} on Physiological Activites of the Acrosome Reaction on Spermatozoa

Jae-Ho Chang, Yung-Keun Oh*, In-Ho Choi, Noh-Pal Jung+, Hyung-Cheul Shin‡

Department of Biology, College of Sciences, Yonsei University, Wonju 220-710;
+Department of Biology, College of Sciences, Yonsei University, Seoul 130;
‡Department of Phisiology, College of Medicine, Hanlim University, Chunchun 200-702, Korea

It has been known that spermatozoa should obtain their fertilizing ability through capacitation and acrosome reaction, and that in these processes of fertilization, Ca^{2+} plays an important role for their conjugation. Therefore the present study has been designed in order to clarify the effect of fluctuation of the media Ca^{2+} level and the intracellular concentration of the spermatozoa on the acrosomes. During the incubation of spermatozoa, a considerable fluctuation in the media Ca^{2+} level has been observed after the BSA administration and the media concentration of Ca^{2+}. It is deduced that these fluctuation rates may have an effect on the acrosome reaction. The fluctuation of K^+ flux has been observed in accordance with the incubation period over time, and it's concentration seems to be closely related with the acrosomal reaction. The respiratory exchange rate (RER) of the spermatozoa is kept more regular in the BSA and $CaCl_2$ administration groups than the non-administration group. Based on the experimental findings, it is possible to deduce a hypothesis from these findings that physiological activities of the acrosome reaction are not functionally related to the media Ca^{2+} level and the intracellular influx of Ca^{2+} concentration, although Ca^{2+} plays an important role as a stimulating factor in the acrosome reaction.

KEY WORDS: Acrosome Reaction, BSA, Ca^{2+} Influx, K^+, RER, Spermatozoa

It is known that the sperm should obtain capacitation as a prerequisite for fertilization in mammals including man. It has also known that the acrosome reaction for capacitation was induced by Ca^{2+} influx from the extracellular fluid into the spermatozoa (Crichton *et al.*, 1993: Fraser and McDermott, 1992: Tesarik *et al.*, 1992), which plays an important role in inducing capacitation. In the process of this calcium influx, the enzymatic release and the oolemmal changes which play important roles for conjugation between the spermatozoa and the ova (Babcock *et al.*, 1979: Fraser, 1987: Magnus *et al.*, 1990). Bedford, 1983, Yanagimachi and Usui (1974) demonstrated that the mammalian spermatozoa ejaculated from the reproductive tract usually obtain capacitation and then they are able to get dynamic motility and ultimately conjugate with the egg plasma membrane. According to the experimental results of Liu and Gadon (1992) such an acrosome reaction and hyperactivated motility may be closely related with Ca^{2+}, and especially such a hyperactivated motility of the spermatozoa can be an important guide post for revealing a perfect capacitation level (Fraser, 1977: Yanagimachi, 1978), and only the spermatozoa

*To whom correspondence should be addressed.

which pass through all the guide posts are able to get capacitation. When guinea pig spermatozoa are primarily incubated in the media without Ca^{2+} and then are secondarily followed in the media with Ca^{2+}, the capacitation is remarkably facilitated. This increase may indicate that the Ca^{2+} is a essential factor for capacitation in the spermatozoa (Yanagimachi and Usui, 1974). The experimental results similar to the above have also been reported in the hamster and mouse (Bolanos *et al.*, 1983: Yanagimachi, 1978). On the other hand, Fraser (1987) have reported that the capacitation became weakened in spermatozoa incubated in the media with Ca^{2+} from the begining. Based on the above experimental results on the Ca^{2+} level as an important factor influencing capacitation, the present study was designed in order to 1) clarify the effect of Ca^{2+} concentration changes on capacitation of the spermatozoa during the early stage including washing procedure for spermatozoa, and 2) observe Ca^{2+} influx and function in the acrosomes, 3) confirm the correlation between the intra- and extracellular level of Ca^{2+} concentrations, and K^+ and Ca^{2+} influx that play an important role in the acrosome reaction and the plasma membrane, and lastly, 4) examine whether or not during spermatozoa incubation the oxygen and the carbon dioxide respiration rate could be applied to acrosomal analysis for evaluating the capacitation.

Materials and Methods

Composition of the culture media

H-BWW (HEPES-buffered Biggers, Whitten and Whittingham) media were used in the present study. They were divided into two application groups: one applied to 3mg/ml Bovine Serum Albumin (BSA; Sigma Chemical Company, St Louis, MO) and followed by sperm washing procedures, and the other not applied to the BSA. The H-BWW media used for sperm washing were administered with 1.71 mL $CaCl_2$ solution and thereafter were divided into 9 different kinds of the $CaCl_2$ concentrations (0 mM, 0.40 mM, 0.90 mM, 1.25 mM, 1.80 mM, 3.60 mM, 5.20 mM, 7.00 mM, and 8.00 mM) respectively.

Preparation of sperm samples

Human sperm ejaculates from normal healthy volunteers were obtained by masturbation. Only samples demonstrating normal semen analysis (sperm count: $>40 \times 10^6$ cells/mL; semen volume:>1.5 mL; motility:>70%) were used for experimentation. After liquefaction at 37°C for 15 minutes, seminal plasma was removed by laying H-BWW containing 3 mg/mL BSA and centifuging at $500 \times g$ for 5 minutes. The sperm pellet was overlaid with 2mL H-BWW containing 3 mg/mL BSA and incubated in a test tube inclined at 45 degrees for 15 minutes to enable the motile sperm to swim up out of the pellet.

Ca^{2+} measurement in the medium

The method used for assaying Ca^{2+} in medium was a modification of the "Determination of calcium". The experiment avoided the troublesome clogging of the burner by adding organic solvents to the diluted mixture and by removing proteins prior to burner aspiration. Lanthanium chloride ($LaCl_3.7H_2O$) was dissolved in distilled water and diluted so as to make solutions that were 0.5% lanthanium (w/v). Pure pulverized $CaCl_2$ was dissolved in distilled water and diluted with distilled water to concentrations of 1, 3, 5, 7, 9, 12, 15, 17 and 20 ppm of calcium respectively. A Perkin-Elmer 303 atomic absorption spectrophotometer using a Perkin-Elmer calcium cathode source was used throughout. The diffraction grating was set to allow peak energy of the 422.7 mμ resonance line of calcium. The scale was used at 1, the response time at 0.1, and the slit set at 0.4. Air was used at a pressure of 26 psi and acetylene at a pressure of 10 psi. The diluted specimens and standards were then aspirated into the burner atomizer via a polyethylene tube connected to the atomizer capillary and 3 independent readings were taken of each solution as a percentage of the absorption counter (for review, see Trudeau and Freier, 1967).

Intracellular Ca^{2+} measurement in the spermatozoa

The method used for assaying Ca^{2+} in the sperm cell was a modification of the "Internalization of the metallochromic Ca^{2+} indicators in mammalian cells" technique developed by Winer and Scarpa (1985). Sperm cells were loaded with an indicator by simply incubating a dense cell suspension in normal physiological saline containing 10 mM glucose and a nearly saturated solution of the indicator. After sperm washing, the experimental group and the control group were suspended in 1 mL of physiological saline supplemented with 10 mM glucose, and 7 mM arsenazo III at pH 7.6. The sperm cells were then incubated at 22°C for 15 minutes, washed 2 twice in 40 mL of the physiological saline solution (pH 7.4), and resuspended at 5 - 7 $\times$ 10^6/mL. The Ca^{2+} concentration of the sperm cells was measured by a UV/vis spectrophotometer (Hitachi, U-2000) at 660 nm and 690 nm.

K^+ measurement in the medium

The method used for assaying K^+ in the medium was the same as the Ca^{2+} measurement method. Pure pulverized K^+ was dissolved in distilled water and diluted with distilled water to concentrations of 1, 3, 5, 7, 12, 15 and 20 ppm of K^+. The diluted specimens and standards were measured (for review, see Gorman and Thomas, 1980, Trudeau and Freier, 1967).

Triple stain technique

To each sperm suspension, an equal volume of Trypan blue (0.5% in H-BWW) was added and incubated for 15 min at 37°C in a water bath, followed by washing with 10 mL H-BWW and centrifugation at 600 g for 8 min. The resulting pellet was resuspended in 10 mL H-BWW and centrifuged at 600 g for 8 min. The final sperm pellet was fixed with 2 mL 3% glutaraldehyde in H-BWW at 35°C in a water bath. After 20 min, the fixative was removed by washing with distilled water and the sample centrifuged at 600 g for 8 min. The pellet was resuspended in 10 mL distilled water and centrifuged at 600 g for 8 min. The supernatant was then discarded and the remaining pellet resuspended by repeated pipetting and 10 l then smeared onto two microslides and air-dried for 30 min.

The triple stain sequence consisted of incubation in 2.0% Bismark Brown Y for 5 min at 40°C in a waterbath, three brief washings in distilled water, followed by incubation in 0.8% Rose Bengal for 27.5 min at room temperature. After three washings in distilled water, sperm were then dehydrated in graded alcohol (50, 75, 90, 95%), cleared in Xylene and examined double-blind, under oil-immersion brightfield optics at 1000 $\times$ magnification by one investigator. For estimation of the acrosome reaction, 400 morphologically normal sperm were evaluated in randomly selected fields and the proportion of viable acrosome-reacted sperm was determined and expressed as a percentage of the viable sperm.

Four staining patterns were classified according to Talbot & Chacon:

(i) Live unreacted cells are unstained by trypan blue with pink acrosomal region and brown postacrosomal region.

(ii) Live reacted cells are also unstained by trypan blue, but the acrosome region is unstained and the postacrosomal region remains brown.

(iii) Dead reacted cells are stained with trypan blue so that the postacrosomal region is a dark blue-black and the acrosomal region is unstained.

(iv) Dead unreacted cells have an acrosomal region that appears a slightly darker pink than the live unreacted cells, and the postacrosomal region stains dark blue-black.

Statistical analysis

The results were analyzed by ANOVA and Regression analysis. Values were considered statistically significant when $P < 0.05$.

Results

Effect of Ca^{2+} in the medium on spermatozoa activity

In the BSA (0.3 mg/mL) treated-group, there was a remarkable increase of Ca^{2+} concentration compared with the control group (Table 1). The acrosome reaction rate was increased in the

Table 1. The Reduction of Ca^{2+} concentration in medium following Ca^{2+} concentration changes in human sperm suspensions incubated in media with and without BSA (0.3 mg/ml) for 30 min or 120 min.

	Medium				
	Incubation	Reduction of Ca^{2+} con. in medium			
Sperm-washing	[Ca^{2+} con.]	[Time]	[ppm]	[Time]	[ppm]
0.3 mg/ml BSA	0.00 mM	*30 min	0.03±0.04	**120 min	-0.13±0.03
1.71 mM Calcium-chloride	0.40 mM		-0.49±0.05		-0.73±0.24
	0.90 mM		-0.38±0.12		-0.49±0.18
	1.25 mM		-1.10±0.04		-1.65±0.11
	1.80 mM		-4.08±0.55		-5.82±0.82
	3.60 mM		-22.41±2.56		-22.03±2.66
	5.20 mM		-18.69±0.51		-28.79±3.57
	7.00 mM		-20.18±0.80		-17.88±5.97
	8.00 mM		-16.44±1.53		-3.17±0.82
Free BSA	0.00 mM	*30 min	0.10±0.08	**120 min	0.37±1.20
1.71 mM Calcium-chloride	0.40 mM		-0.40±0.22		-0.65±1.43
	0.90 mM		0.22±0.34		-1.23±0.27
	1.25 mM		-0.66±0.06		-1.58±0.93
	1.80 mM		-1.47±0.07		-3.05±2.55
	3.60 mM		-6.16±0.53		-7.23±2.18
	5.20 mM		-11.84±0.60		-11.69±2.59
	7.00 mM		-8.46±1.04		-15.18±6.96
	8.00 mM		-22.00±1.00		-13.52±8.15

*P<0.0001, **P<0.0001, Values are means±SEM.

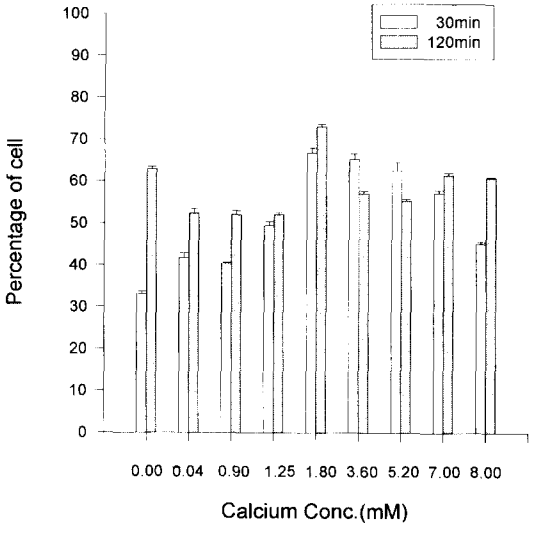

Fig. 1. Acrosome reacction pattern in human sperm suspensions incubated in media containing 0.3 mg/ml BSA for 30 min or 120 min. P<0.05.

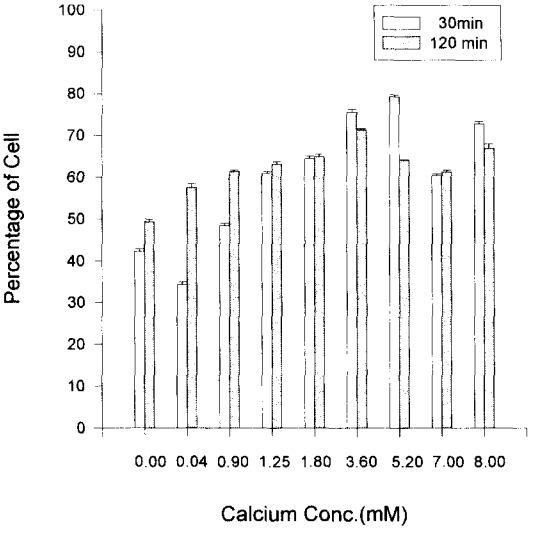

Fig. 2. Acrosome reaction pattern in human sperm suspensions incubated in media without BSA 30 min or 120 min. P<0.05.

Table 2. Ca^{2+} absorbance of sperm cell following Ca^{2+} concentration changes in human sperm suspensions incubated in media with and without BSA (0.3 mg/ml) for 30 min or 120 min.

Medium					
	Incubation	Ca^{2+} Absorbance of sperm cell			
Sperm-washing	[Ca^{2+} con.]	[Time]	[Absorbance]	[Time]	[Absorbance]
0.3 mg/ml BSA	0.00 mM	*30 min	0.8690±0.0412	**120 min	0.1672±0.0254
1.71 mM Calcium-chloride	0.40 mM		0.7119±0.0391		0.1479±0.0227
	0.90 mM		0.3953±0.0533		0.1462±0.0191
	1.25 mM		0.2876±0.0452		0.1595±0.0156
	1.80 mM		0.1842±0.0145		0.2500±0.0151
	3.60 mM		0.1700±0.0231		0.2723±0.0168
	5.20 mM		0.1304±0.0103		0.1968±0.0197
	7.00 mM		0.0665±0.0145		0.3517±0.0223
	8.00 mM		0.0336±0.0178		0.1854±0.0102
Free BSA	0.00 mM	*30 min	0.3951±0.0134	**120 min	0.4114±0.0268
1.71 mM Calcium-chloride	0.40 mM		0.3486±0.0309		0.1384±0.0307
	0.90 mM		0.2066±0.0230		0.4341±0.0468
	1.25 mM		0.1858±0.0150		0.4329±0.0191
	1.80 mM		0.1455±0.0189		0.2919±0.0179
	3.60 mM		0.1404±0.0193		0.4094±0.0036
	5.20 mM		0.0503±0.0098		0.8452±0.0451
	7.00 mM		0.0318±0.0198		0.4344±0.0195
	8.00 mM		0.0041±0.0029		.04893±0.0017

*$P<0.0001$, **$P<0.0001$, Values are means±SEM.

higher concentration change of Ca^{2+} (Fig. 1, 2) which caused a significant effect of Ca^{2+} in the medium and the BSA treatment.

Effect of intracellular Ca^{2+} on the spermatozoa activity

A significant relationship was found between the intracellular Ca^{2+} level and the acrosome reaction rate by means of the spectrophotometer analysis measuring the absorption rate of intracellular Ca^{2+} (Table 2).

Effect of K^+ on the spermatozoa activity

At the 120 min incubation, remarkably most of the K^+ transfer occurred in the concentration in which the acrosome reaction took place most frequently (Table 3, Fig. 1, 2). The acrosome reaction rate increased at the condition of large scale of fluctuations of K^+ and thus indicated that the Ca^{2+} concentration changes in the culture medium and the acrosome reaction rate seem to influence the K^+ changes.

Observation on the acrosome reaction

When the treated group and the control group were compared, there was no significant difference in their acrosome reaction rates (Fig. 1, 2). In the BSA group (experimental group), at the 30 min the acrosome reaction occurred at 3.6 mM concentration, while at the 120 min maximum acrosome reaction occurred is 1.8 mM concentration (Fig. 1).

In the free BSA group, at both the 30 min and the 120 min, the maximum reaction rate was observed in 3.6 mM (Fig. 2). In the BSA group, at the 30 min incubation, the acrosome reaction once more occurred highly in the 7 mM concentration althrough the Ca^{2+} concentration was kept high. This indicates that Ca^{2+} functions as a parameter for influencing the acrosome reaction rate through transport of Ca^{2+} from the incubation media.

Table 3. The K^+ concentration changes in medium following Ca^{2+} concentration changes in human sperm suspensions incubated in media with and without BSA (0.3 mg/ml) for 30 min or 120 min.

	Medium				
	Incubation	Reduction of K^+ con. in medium			
Sperm-washing	[Ca^{2+} con.]	[Time]	[ppm]	[Time]	[ppm]
0.3 mg/ml BSA	0.00 mM	*30 min	0.25±0.19	**120 min	3.08±0.33
1.71 mM Calcium-chloride	0.40 mM		2.83±0.64		1.05±0.14
	0.90 mM		-0.41±0.86		2.81±0.11
	1.25 mM		0.34±1.49		0.82±0.06
	1.80 mM		0.55±0.06		1.08±0.13
	3.60 mM		4.79±5.81		0.39±0.02
	5.20 mM		0.48±0.08		0.39±0.03
	7.00 mM		0.13±0.01		-0.01±0.04
	8.00 mM		0.58±0.06		-0.34±0.31
Free BSA	0.00 mM	*30 min	1.55±0.40	**120 min	0.10±0.02
1.71 mM Calcium-chloride	0.40 mM		0.16±0.08		2.27±0.13
	0.90 mM		1.01±0.17		0.69±0.20
	1.25 mM		4.23±0.31		0.54±0.07
	1.80 mM		5.20±0.08		0.31±0.10
	3.60 mM		5.23±0.03		0.26±0.05
	5.20 mM		3.24±0.13		0.72±0.06
	7.00 mM		1.26±0.04		-0.43±0.93
	8.00 mM		-0.08±0.26		1.03±0.75

*P<0.0004, **P<0.0001, Values are means±SEM.

In the free BSA group, at the 5.2 mM concentration, the maximum acrosome reaction rate was found, and in the BSA group, at the three concentration ranges between 1.8 mM and 5.2 mM, the highest acrosome reaction rate occurred although differences were recognized to exist according to the BSA administration and the variety of Ca^{2+} concentrations, especially when the maximum acrosome reaction rate appeared in the 3.6 mM Ca^{2+} concentration (Fig. 1, 2).

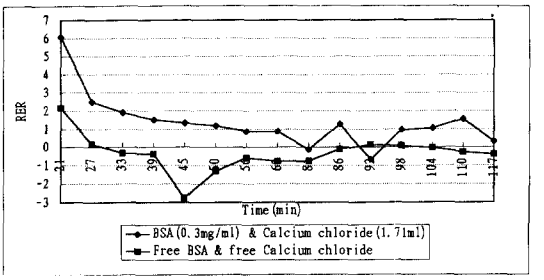

Fig. 3. Effect of 0.3 mg/ml BSA and $CaCl_2$ on RER for 21 min to 120 min. P<0.05.

Effect of BSA and $CaCl_2$ on respiration rate of spermatozoa during incubation (RER)

In the spermatozoa in the incubation media treated with the BSA (0.3 mg/ml) and the $CaCl_2$ (1.71 mM) which were swimed up, the respiration rate (RER) was generally higher than that of the spermatozoa in the control media. Within 81 min of the incubation, differece between both the treated and control group became small: the width of the difference was temporarily observed to be reversed at the 92 min (Fig. 3).

Discussion

In the present study, the H-BWW media was used from washing procedure of the spermatozoa

to culturing them and thus it was certain that the fluctuation of Ca^{2+} concentration was not influenced by any other possible salt source. However, it is of interest that the Ca^{2+} fluctuation and the physiological metabolism, and the respiration exchange rate (RER) can be quantitatively analyzed through washing procedures using 0.3 mg/mL BSA (Pilikian and Mimouni, 1988).

Ca^{2+} fluctuation rates in the medium indicate that the acrosome reaction may be activated in accordance with the extracellular Ca^{2+} concentration, the fluctuation rate of which can be influenced more largely than those of the fluctuation size.

Analyzing the intra-medium fluctuation of Ca^{2+}, at the 3.6 mM, showed that in spite of the intra-medium fluctuation of Ca^{2+} and the acrosome reaction occurred highly, the intracellular absorbance of Ca^{2+} appeared low. This decreasing phenomena can be considered to be due to the Ca^{2+} influx into the mitochodria, which influences the motility of the spermatozoa (Breitbart *et al.*, 1985) and/or due to the excessive concentration of Ca^{2+}-ATPase during the acrosome reaction, which influences the spermatozoan motility (Fraser and McDermott, 1992).

The absorbance rate of the intra-media K^+ was analyzed in order to clarify the intracellular K^+ concentration controlling the intracellular salts level and to observe whether these controlling factors could maintain the cellular volume and influence the acrosome reaction and their motility or not. There are some experimental reports to clarify the induction mechanism of K^+ conductance in the plasma membrane: due to the ionized Ca^{2+} influx into the plasma membrane (Gorman & Thomas, 1980), due to the proportional increment of K^+ current toward the extracellular space. It is suggested by the experimental results of the present study that in the BSA group, intra-media Ca^{2+} concentrations and high fluctuation rate were generally synchronized with the high acrosome reaction rate and the high K^+ concentration.

Observing the respiratory exchange rate (RER) in the BSA group, the respiration rate of the spermatozoa was stabilized at 0-2 (exchange rate of oxygen and carbon dioxide) within 30 min after tissue culture. This stabilization persisted for 120 min to reveal that the respiration exchange was maintained definitely and which suggested that the stable transfer of Ca^{2+} concentration may be closely related to the spermatozoan respiration.

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Ca^{2+}이 정자 첨체반응의 생리적 활성에 미치는 영향

장재호 · 오영근 · 최인호 · 정노팔 · 신형철[+]
(연세대학교 생물학과, [+]한림대학교 의과대학 생리학교실)

정자가 수정을 하기 위해서는 수정능 획득(capacitation)과 첨체반응(acrosome reaction)을 거쳐 수정능력(fertiliting ability)을 획득하여야 한다. 이러한 수정과정에서 Ca^{2+}이 중요한 인자로 작용을 하는데 본 실험에서는 정자배양시 배지내 Ca^{2+} 변동량과 정자내 Ca^{2+} 농도가 정자첨체부에 어떠한 영향을 미치는가를 알아보기 위한 실험을 하여 다음과 같은 결론을 얻었다. 정자를 배양할 때 배지내 Ca^{2+}의 변동량은 정자세척시 BSA (bovine serum albumin) 처리여부와 배양배지내 Ca^{2+} 농도에 따라 많은 영향을 받았으며 Ca^{2+}의 변동량 자체보다는 각각의 배양농도에 따른 Ca^{2+}의 변동율이 첨체반응에 영향을 주었고, 배양시 정자세포내로 유입된 Ca^{2+}이 배지내 Ca^{2+} 농도에 따라 증가하지는 않지만 첨체반응에는 상당히 유의적인 영향을 미친다고 사료된다. K^+의 이동량은 유입되는 Ca^{2+} 농도와 직접적인 연관성은 없지만, 배양시간에 의해 영향을 받는다는 것과 K^+ 농도 변화와 첨체반응과는 상당히 유의적인 관계가 있음을 나타냈다.

정자배양시 정자호흡의 경우 BSA(0.3 mg/mL)와 $CaCl_2$(1.71 mM)를 처리한 배지로 배양시킨 바 비처리시보다 산소와 이산화탄소의 소모량과 호흡상(respiratory exchange rate)이 일정하게 나타났다. 이상의 실험결과로 보아 첨체반응에서 Ca^{2+} 농도가 중요한 인자로 영향을 미치지만, 배양배지내 Ca^{2+} 변동과 정자세포내로 유입되어 존재하는 Ca^{2+} 농도와는 비례적인 관계가 성립되지 않는다고 볼 수 있다.