

POTASSIUM IONS IMPROVE THE PROGESTERONE-INDUCED $[Ca^{2+}]_i$ INCREASE AND ACROSOME REACTION AS WELL AS THE POTASSIUM-INDUCED $[Ca^{2+}]_i$ INCREASE IN HUMAN SPERMATOZOA

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ABSTRACT

Ejaculated sperm undergo a series of physiological changes in the female reproductive tract to become capable of fertilization. These changes, called capacitation can be induced in vitro in appropriate media and culminate in hyperactivated motility and the ability of the sperm to acrosome-react. These downstream effects of capacitation are mediated by increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Exposure of sperm to progesterone (P_4) and potassium (K^+) induce an increase in $[Ca^{2+}]_i$. The effects of incubating sperm in media containing different potassium concentrations on the P_4 -induced $[Ca^{2+}]_i$ increase and AR as well as the effect of K^+ on $[Ca^{2+}]_i$ increase were investigated. Swum-up spermatozoa were capacitated for about 6 h in culture media containing either 5.4 (control), 25 or 116.4 mM K^+ before being stimulated with P_4 . P_4 evoked a biphasic $[Ca^{2+}]_i$ response in spermatozoa incubated in each of the media. The amplitude of the transient P_4 -induced increase in $[Ca^{2+}]_i$ and the percentage of P_4 -induced AR were significantly lower in sperm incubated in media with very low or very high K^+ concentration. Absence of K^+ in the incubating medium significantly inhibited the amplitude of both the P_4 - and K^+ -induced $[Ca^{2+}]_i$ increases. Application of K^+ after P_4 application evoked a second biphasic $[Ca^{2+}]_i$ response. The results suggest that i) the presence of K^+ in the incubating medium is important in the K^+ -induced $[Ca^{2+}]_i$ increase as well as the P_4 -induced $[Ca^{2+}]_i$ increase and AR, ii) human sperm capacitates better in an incubating medium with a K^+ concentration around that of oviductal fluid and iii) P_4 - and K^+ utilize different channels for inducing Ca^{2+} influx.

INTRODUCTION

The mammalian oviduct provides the microenvironment for several reproductive events, such as fertilization and preimplantation embryonic development (Roblero *et al*, 1988). The human oviductal fluid has been shown to contain high concentrations of chloride and potassium but low concentrations of calcium relative to the range of normal human serum values. Sodium

and magnesium concentrations in the oviductal fluid are similar to serum levels. The potassium concentration in the human oviduct can reach a level five times that found in peripheral plasma during the pre-ovulatory period (Borland *et al.*, 1980). Because the majority of the processes experienced by the spermatozoa in the acquisition of its fertilizing ability take place in the oviduct, it is reasonable to believe that changes

in the ionic composition of the oviductal fluids could influence the ability of the spermatozoa to mature and fertilize (Roblero *et al.*, 1988). Ion environment and ionic fluxes through membrane are thought to be important in the spermatozoa's maturation, capacitation, and initiating the process of gamete interaction (Shi and Ma, 1998). A variety of ion channels have been detected in human (Shi and Ma, 1998; Chan *et al.*, 1997) and rat (Chan *et al.*, 1997) spermatozoa plasma membranes. Indeed, K^+ -selective and TEA^+ -sensitive channels have been identified in spermatogenic cells (Hagirawa and Kawa, 1984) and in bilayers containing rat and human sperm plasma membranes (Chan *et al.*, 1997).

In vitro studies in mouse and bovine sperm populations have shown that capacitation is accompanied by a plasma membrane hyperpolarization that is thought to be mediated in part, by an enhanced K^+ permeability (Zeng *et al.*, 1995). It has been established that increased cytosolic pH (pH_i) promotes metabolic and swimming activity of bull sperm and that intracellular alkalinization results from elevated extracellular K^+ , presumably as a consequence of membrane depolarization (Babcock *et al.*, 1983). It has also been shown that a persistent but reversible increase in $[Ca^{2+}]_i$ accompanies the increase in pH_i that similarly results from treatment of ram sperm with elevated $[K^+]$ in alkaline medium (Babcock and Pfeifer, 1987). Various inwardly (Munoz-Garay *et al.*, 2001; Avededo *et al.*, 2006) and outwardly rectifying (Navarro *et al.*, 2007) K^+ channels have been described in both spermatogenic cells and sperm. These have been said to contribute to the capacitation-associated hyperpolarization.

It has been reported that sperm survival in a culture medium containing 25 mM K^+ is significantly higher compared with media that contained 4.7 or 15 mM K^+ . Furthermore, a greater percentage of sperm acrosome reacted in 25 mM K^+ compared with 4.7 and 15 mM K^+ ; also the time taken to achieve 20% of AR was significantly shorter in 25 mM K^+ compared with 4.7 and 15 mM K^+ (Roblero *et al.*,

1988; Roblero *et al.*, 1990). The ability of sperm to penetrate oocytes is higher when sperm were incubated 25 mM K^+ than in 4.7 mM K^+ and it has been suggested that Na^+,K^+ -ATPase activity and resulting K^+ influx are important for mammalian sperm AR (Mrsny and Meizel, 1981). It has been reported in mouse sperm that acrosome loss and hyperactivated motility were significantly lower in media with very low or very high K^+ concentrations but, after alteration to control medium values, increased to levels similar to those obtained with control samples. It is proposed that the relatively high K^+ concentrations found in female tract fluid (approximately 20-30 mM) may serve to modulate fertilizing potential of sperm *in vivo* (Fraser, 1983). K^+ (1.25-20) has been shown to induce a dose-dependent increase in $[Ca^{2+}]_i$; similar to that observed with P_4 . It appears that human sperm plasma membrane possesses different Ca^{2+} channels responsive to P_4 and K^+ (Kumar *et al.*, 2000).

The purpose of this study was to investigate the effects of different extracellular K^+ concentrations on the P_4 -induced $[Ca^{2+}]_i$ increase and AR as well as the K^+ -induced $[Ca^{2+}]_i$ increase in human spermatozoa.

MATERIALS AND METHODS

Materials

Progesterone [4-pregnene-3,20-dione], salts for the preparation of Earle's balanced salt solution (EBSS), Digitonin, Ethylene glycol-bis (β -amino-ethylether)-N,N,N',N'-tetraacetic acid (EGTA), A23187, dimethylsulphoxide (DMSO), lectin from *Pisum sativum* (FITC-labelled), 4-(2-hydroxyethyl)1-piperazine ethanesulfonic acid (HEPES), choline chloride, were all from SIGMA-ALDRICH. Fura-2 AM was from Molecular Probes, and bovine serum albumin (BSA) was from JRH Biosciences.

Preparation and Capacitation of Sperm

All donors were recruited at the Birmingham Women's Hospital (HFEA centre number 0119), in accordance with the Human Embryology Authority Code of Practice. Human ejaculated spermatozoa were obtained from normal

healthy donors of proven fertility by masturbation. After semen liquefaction (approximately 30 min), motile spermatozoa were harvested by swim-up (Mortimer, 1994). Briefly 1 ml of a HEPES-buffered saline (containing NaCl (150 mM), KCl (5 mM), CaCl₂ (2 mM), MgCl₂ (1 mM), glucose (10 mM) and HEPES (10 mM)) of pH of 7.4, or (sEBSS) (containing NaCl (116.4 mM), KCl (5.4 mM), CaCl₂ (1.8 mM), MgCl₂ (1 mM), glucose (5.5 mM), NaHCO₃ (25 mM), Na pyruvate (2.5 mM), Na lactate (19 mM), MgSO₄ (0.81 mM) + 0.3% BSA), of pH 7.4, was underlaid with 0.3 ml of liquefied semen in falcon tubes. The tubes were then incubated for 1 hour at 37°C, in 5% CO₂. After 1 hour the upper 0.7 ml of the medium (containing the motile fraction of spermatozoa) of all the tubes were collected into a 15 ml Blue max tube (Becton Dickinson, USA) using a sterile transfer pipette. The concentration of the collected spermatozoa were assessed using a Neubauer counting chamber according to the WHO methods (WHO, 1999) and adjusted to 6 x 10⁶ cells per ml in the appropriate media. 2 ml and 200 µl aliquots of the spermatozoa were incubated for at least 6 hours at 37°C, 5% CO₂, for Ca²⁺ measurement and AR experiments respectively.

Effects of extracellular concentrations of potassium on the P₄-induced [Ca²⁺]_i increase and Acrosome Reaction

After sperm had been capacitated, as described above, aliquots (2 ml) of sperm suspended in sEBSS containing 0.3 % BSA but different concentrations of KCl were prepared for [Ca²⁺]_i determination. Three concentrations were tested: 5.4 mM, (control), 25 and 116.4 mM K⁺ (treatments). In each treatment increasing concentrations of extracellular K⁺ were compensated by proportionally lowering the NaCl concentration to ensure that the osmolality of the modified sEBSS was kept at 285-295 mosm/kg.

Measurement of [Ca²⁺]_i

2ml aliquots of sperm were prepared for [Ca²⁺]_i determination by labelling with acetoxymethyl ester of fura-2 (1 µM final extracellular concen-

tration) for 12 minutes at 37°C, 5% CO₂. After dye loading each 2 ml sample was centrifuged at 500g for 5 minutes. The supernatant was discarded and the pellets were resuspended in the appropriate media ie 2 ml sEBSS containing 0.3% BSA and the different concentrations of KCl. These were incubated for 17 minutes at 37°C, 5% CO₂ before was measured [Ca²⁺]_i. Fluorimetric studies were conducted using a Perkin-Elmer LS50B luminescence spectrometer. Fluorimetric [Ca²⁺]_i measurements were performed using an excitation wavelength pair of 340/380 nm and an emission wavelength of 510 nm. Fluorimetry was performed in a methylacrylate cuvette magnetically stirred and warmed to 37°C in a heated cuvette holder. Sufficient time (2-5 min) was allowed for the temperature of the sperm suspension to reach 37°C before measuring [Ca²⁺]_i. P₄ (3.2 µM, final) was added at 400 sec and 20 µM digitonin was added at approximately 800 sec after the start of each experiment. 47 µM of EGTA was added at approximately 900 sec after the start each experiment. The sequential addition of digitonin and EGTA were done to facilitate determination of "calculated [Ca²⁺]_i" as previously described (Grynkiewicz *et al.*, 1985), using a K_d of 285 nM for fura-2 at 37°C (Grodén *et al.*, 1991).

Assessment of Progesterone-induced Acrosome Reaction

After capacitation as described above, 200 µl aliquots of spermatozoa suspended in the appropriate media were stimulated with either progesterone (final concentration of 3.2 mM) or A23187 (10 mM) or solvent control (0.05 % DMSO). After an incubation period of 1 hour, spermatozoa were centrifuged at 500g for 5 minutes. The supernatant was removed and the spermatozoa resuspended in 0.5 ml of hypo-osmotic swelling (HOS) medium (0.74% sodium citrate, 1.35% fructose in double-distilled H₂O). After 45 minutes incubation in HOS media, the spermatozoa were centrifuged for 5 minutes at 500 g. The supernatant was removed leaving a minimum volume of HOS (30 µl) for resuspension. Resuspended pellets in remaining

HOS were smeared on microscopic slides (duplicate slides) previously coated with 10% poly-L-lysine solution and air-dried.

The cells were then permeabilized in methanol for 30 seconds. About 15ml of FITC-labelled *Pisum sativum* agglutinin (FITC-PSA) in PBS was spread on each slide and incubated for 45 minutes in a humid chamber at 37°C. Slides were then washed in a constant flow of mains water for 5 minutes before air drying and mounting with hydromount. Fluorescence microscopy was used to evaluate acrosomal status; slides were scored blind and only viable (curly tailed) spermatozoa were scored (Aitken *et al.*, 1993). Acrosomal status was assessed as described elsewhere (Mendoza *et al.*, 1992). A total of 200 spermatozoa were scored for each treatment (100 per slide). Progesterone-induced AR was represented as the percentage of stimulation by normalizing to a maximum stimulation by DMSO treatment for each individual experiment, using the equation:

$$\text{Stimulation (\%)} = \left[\frac{(R - \text{DMSO})}{\text{ionophore} - \text{DMSO}} \right] \times 100$$

Where R is the percentage AR in the experimental incubation, DMSO is the percentage AR in the parallel vehicle control and ionophore is the percentage AR in the parallel ionophore-treated incubation.

Effects of extracellular potassium on the K⁺- and P₄-induced [Ca²⁺]_i

The investigations centred on K⁺- and P₄-induced [Ca²⁺]_i increase after incubation in one of two media: complete sEBSS (which contained 5.4 K⁺) (control), and K⁺-free sEBSS (KCl was replaced with choline chloride). Aliquots (2 ml) of the sperm suspension were incubated for at least 6 hours at 37°C, 5% CO₂, after which the spermatozoa were labelled for [Ca²⁺]_i measurement.

Measurement of [Ca²⁺]_i

[Ca²⁺]_i measurement was as described above, but in another set of experiments, P₄ was added at 500s and KCl or choline chloride was added

at 800s. This was done to ascertain whether the increase induced by K⁺- and P₄ utilized the same channels.

Statistical Analysis

Data are expressed as mean ± SEM for n experiments as indicated in figure legends. Statistical analysis was performed with t-test for paired or unpaired data when comparing classes. Statistical significance was set at P < 0.05.

RESULTS

The effects of [K⁺]_o on the P₄-induced [Ca²⁺]_i response in human sperm was evaluated. In this study, swum up spermatozoa were incubated for about six hours media containing three different K⁺ concentrations (5.4, 25 and 116.4 mM). The effects of K⁺ concentrations on the P₄-induced [Ca²⁺]_i response in human sperm is shown in Fig.1. P₄ induced a biphasic response in spermatozoa incubated in each of the three media. The results show that [K⁺]_o influenced the amplitude of the transient P₄-induced [Ca²⁺]_i increase. The amplitude of the transient P₄-induced [Ca²⁺]_i increase was higher in spermatozoa incubated in the medium containing 25 mM K⁺ than in those incubated in the control medium and those incubated in the medium containing 116.4 mM K⁺. The amplitude of the transient P₄-induced [Ca²⁺]_i increase of spermatozoa incubated in the medium containing 25 mM K⁺ though, not significantly different from that of those incubated in the control medium, was significantly (p < 0.05) different from that of those incubated in medium containing 116.4 mM K⁺ (Fig. 1 insert). There was however, no significant (p < 0.05) difference between the transient P₄-induced [Ca²⁺]_i response of spermatozoa incubated in the control medium and that of those incubated in the medium containing 116.4 mM K⁺. The P₄-induced transient [Ca²⁺]_i increase of cells incubated in the medium containing 116.4 mM K⁺ peaked significantly later (18.49 ± 0.52 s) than those incubated in the control medium (14.18 ± 0.74 s) and the medium containing 25 mM K⁺ (14.45 ± 0.95 s). There was no significant (p < 0.05)

difference between the time of peaking of the transient in the control medium and that containing 25 mM K^+ .

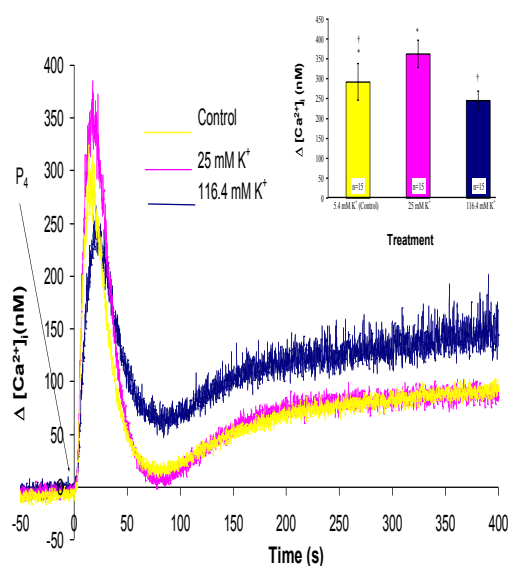


Fig. 1. The effects of different concentrations (5.4 mM (control), 25 mM, 116.4 mM) of K^+ on P_4 -induced $[Ca^{2+}]_i$ increase in human spermatozoa. To these samples P_4 (3.2 mM) was added at 400s. Insert: Average transient $[Ca^{2+}]_i$ increase in response to P_4 in the presence of the different concentration (5.4 mM, 25 mM, 116.4 mM) of K^+ . The error bars represent the SEM, and the number of experimental replicates is denoted within each bar. Different symbols indicate differences in significance ($p < 0.05$)

Having investigated the effects of incubating sperm in the three different media on the P_4 -induced $[Ca^{2+}]_i$, their effects on the P_4 -induced AR was also evaluated. The media had a significant influence on the percentage acrosome reaction. The percentage of acrosome reaction was significantly ($p < 0.05$) higher in the medium containing 25 mM K^+ than the control medium and the medium containing 116.4 mM K^+ . That of the control medium was also significantly higher than that of the medium which contained 116.4 mM K^+ (Fig.2).

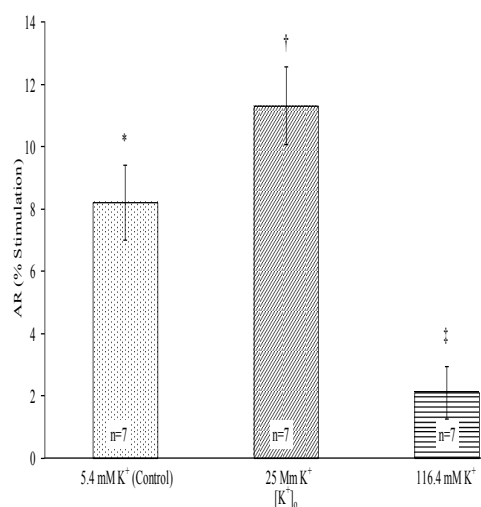


Fig. 2. The effects of different concentrations (5.4 Mm (control), 25 mM, 116.4 mM) of K^+ on P_4 -induced AR. The error bars represent the SEM, and the number of experimental replicates is denoted within each bar. Different symbols indicate differences in significance ($p \leq 0.05$)

Having evaluated the effects of different concentrations of $[K^+]_o$ on the P_4 -induced $[Ca^{2+}]_i$ increase, the effect of incubating sperm in a K^+ -free medium was evaluated. In this study cells were swum-up in K^+ -free sEBSS which had the KCl fraction replaced with choline chloride, cells were resuspended in either the same medium or in complete sEBSS. The transient P_4 -induced $[Ca^{2+}]_i$ increase in sperm incubated in complete sEBSS was significantly higher ($p < 0.05$) than that of those incubated in K^+ -free sEBSS (Fig. 3).

The effect of K^+ on capacitated human spermatozoa was evaluated. Sperm were swum-up in K^+ -free sEBSS and resuspended in same medium or in complete sEBSS. The addition of K^+ (final concentration=25 mM), to the cuvette containing sperm incubated in the control medium induced a biphasic calcium response. There was a rapid elevation of $[Ca^{2+}]_i$ to a peak level which declined to almost the basal level

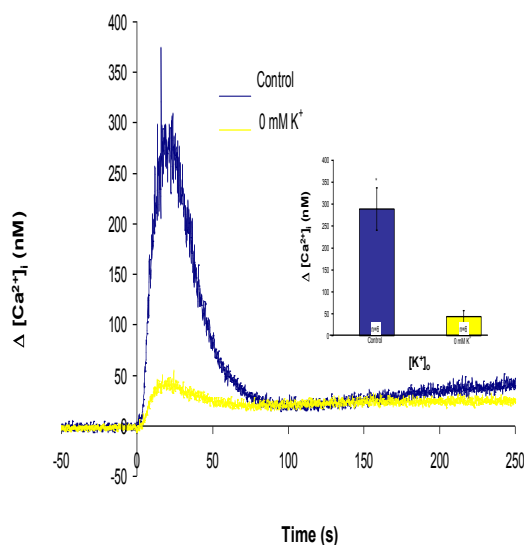


Fig. 3: The effects of incubation in complete sEBSS (control) and K^+ -free sEBSS on the P_4 -induced $[Ca^{2+}]_i$ increase in human spermatozoa. Insert: Average transient $[Ca^{2+}]_i$ increase in response to P_4 of cells incubated in complete sEBSS (control) and K^+ -free sEBSS. The error bars represent the SEM, and the number of experimental replicates is denoted within each bar. Different symbols indicate differences in significance ($p < 0.05$)

then rose to a smaller sustained response. K^+ generated virtually no response in sperm incubated in K^+ -free sEBSS (Fig. 4). To ascertain whether K^+ - and P_4 -induced $[Ca^{2+}]_i$ increase utilized the same channels, K^+ was added 300 s after addition P_4 in the same set of experiments. Both K^+ and P_4 evoked the same Ca^{2+} response even though the amplitude of the transient of the K^+ -induced $[Ca^{2+}]_i$ increase was significantly ($p < 0.05$) lower than that evoked by P_4 (results not shown).

DISCUSSION

The study shows that for both P_4 - and K^+ to induce Ca^{2+} influx in human spermatozoa, there should be some K^+ in the incubating medium. The amplitude of the P_4 -induced $[Ca^{2+}]_i$ increase increased from a minimum in K^+ -free

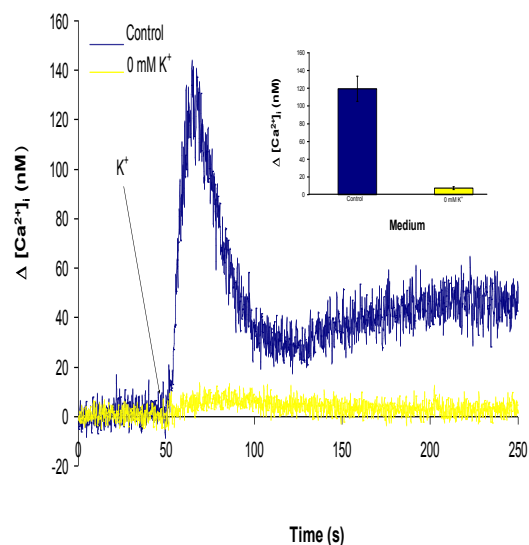


Fig. 4: The effects of K^+ (final concentration=25 mM) on $[Ca^{2+}]_i$ increase in human spermatozoa incubated in control medium (5.4 mM K^+) or K^+ -free medium (0 mM K^+ ; K^+ replaced with choline chloride). Insert: Average transient $[Ca^{2+}]_i$ increase in response to K^+ or choline chloride in the presence of 5.4 mM (control) and 0 mM of K^+ . The error bars represent the SEM. There were 6 experimental replicates. Different symbols indicate differences in significance ($p < 0.05$)

sEBSS to a maximum in the medium containing 25 mM K^+ which is similar to the ionic composition of the fallopian tube fluid (Borland *et al.*, 1980), then dropped significantly in the medium which contained 116.4 mM K^+ , almost similar to the measured concentration of K^+ in both vas deferens and cauda epididymal fluid from one man (Turner, 1979).

Murine, bovine and human sperm intracellular fluids have a high K^+ concentration of between 90 and 120 mM (Babcock, 1983; Zeng *et al.*, 1995; Linares-Hernandez *et al.*, 1998). The K^+ concentration in both human serum (3.6-5.0 mM) and oviductal fluid (21.2 ± 0.24) (Borland *et al.*, 1980) is well below the intracellular concentration of 75 mM (Patrat *et al.*, 2002) or 120 mM (Linares-Hernandez *et al.*, 1998) re-

ported in human spermatozoa. Inasmuch as K^+ ions are at higher concentrations intracellularly than extracellularly, the opening of K^+ channels causes these cations to leave the cell and hyperpolarize the membrane. There is evidence that during capacitation, the membrane potential of mature mammalian sperm hyperpolarizes from approximately -50 mV to -80 mV (Arnoult *et al.*, 1999). Capacitation of mouse and bull spermatozoa is associated with an increased permeability to potassium ions that would be responsible for the observed membrane hyperpolarization (Zeng *et al.*, 1995). This hyperpolarization affects voltage-dependent channels especially the T-type calcium channels present in sperm. Hyperpolarization during sperm capacitation is sufficient to relieve steady-state voltage-dependent inactivation of the T-type Ca^{2+} currents (Arnoult *et al.*, 1996a) recruiting them from an inactivated state to a closed state that can be subsequently activated to initiate AR (Munoz-Garay *et al.*, 2001).

The results of this study suggest that human sperm capacitate better in the medium containing 25 mM K^+ than that containing 116.4 mM K^+ and the control medium. This is consistent with the findings of Roblero *et al.* (1990; 1988). It is possible that the K^+ efflux in human sperm incubated in the medium containing 25 mM K^+ is more efficient compared to that in sperm incubated in K^+ -free sEBSS and sEBSS making the plasma membranes well hyperpolarized thus putting the channels in the closed state to be activated by P_4 . It has been reported that extracellular K^+ at higher levels than the serum level produce a more rapid capacitation and/or AR in hamster sperm *in vitro* (Mrsny and Meizel, 1981). Since the K^+ concentration in the medium containing 116.4 mM K^+ is higher than the measured intracellular K^+ in human spermatozoa, there will be an influx of the ion resulting in depolarization, hence the low amplitude of the transient of the P_4 -induced likely $[Ca^{2+}]_i$ increase. Depolarized membrane potential prevents unregulated Ca^{2+} entry (Munoz-Garay *et al.*, 2001).

The percentage acrosome reaction after P_4 challenge was also influenced by the amount of K^+

present in the incubating medium. The percent AR was significantly higher in spermatozoa incubated in the medium containing 25 mM K^+ than that of spermatozoa incubated in the control medium or the medium containing 116.4 mM K^+ . A finding consistent with that of Roblero *et al.*, (1988; 1990) who found that high K^+ (25 mM K^+) concentration improved the rate of AR and fertilizing ability of human spermatozoa. This could be due to the reasons stated above. The K^+ -efflux dependent hyperpolarization makes the cells more responsive to P_4 thus enhancing the P_4 -induced $[Ca^{2+}]_i$ increases and the subsequent AR.

This report provides evidence that suggest that some amount of K^+ should be present in the incubating medium for P_4 to evoke the $[Ca^{2+}]_i$ response as well as acrosome reaction. The maximum effect could be attained in cells incubated in medium containing K^+ concentration around the concentration found in the fallopian tube and lower in spermatozoa that were incubated in media containing very low or very high K^+ concentrations.

The results also demonstrate that K^+ also induces $[Ca^{2+}]_i$ increase and this effect can only be effected when there is K^+ in the incubating medium. The amplitude of the K^+ -induced $[Ca^{2+}]_i$ increase was significantly higher in sperm incubated in sEBSS compared to those incubated in K^+ -free sEBSS. K^+ virtually did not evoke any response in sperm incubated in K^+ -free sEBSS. This suggests that K^+ -induced $[Ca^{2+}]_i$ increase is evoked when there is K^+ in the extracellular medium. KCl causes depolarization and concomitant increases in $[Ca^{2+}]_i$, through putative VDCC in human spermatozoa (Gonzalez-Martinez *et al.* 2002). The fact that K^+ evoked the $[Ca^{2+}]_i$ increases after the P_4 -induced response, suggests that the two agonists use separate channels to bring about their effects. It has been demonstrated that, when added together P_4 and K^+ generated a response equal to the sum when they are added separately (Kumar *et al.*, 2000) and also the P_4 -induced $[Ca^{2+}]_i$ influx is known to be subject to desensitization, with second and subsequent applications of 3 mM P_4 being ineffective

(Harper *et al.*, 2003). Since the K^+ -induced $[Ca^{2+}]_i$ influx is caused by K^+ depolarization of the plasma membrane (Fraire-Zamora and Gonzalez-Martinez, 2004; Gonzalez-Martinez *et al.* 2002), VDCCs may be involved in this $[Ca^{2+}]_i$ influx.

The results of this study suggest that extracellular K^+ is needed for human sperm function and that, the P_4 -induced AR in human spermatozoa incubated in the medium containing 25 mM K^+ is significantly higher than that of sperm incubated in the control medium. This suggests that the higher $[K^+]$ found in the female tract may play a significant role in capacitation and AR *in vivo*.

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