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List of Abbreviations:

4AP	4-aminopyridine
AR	Acrosome reaction
ATP	Adenosine triphosphate
Ca ²⁺	Calcium
CBTX	Charybdotoxin
cDNA	cDeoxyribonucleic acid
dUTP	deoxyuracil triphosphate
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde phosphate dehydrogenase
HABA	2-(4-hydroxyazobenzene) benzoic acid
HRP	Horseradish peroxidase
HUCH	Human calcium channel
IVF	In vitro fertilization
K ⁺	Potassium
KCNA3	Human voltage gated K ⁺ channel
Man-AR	Mannose stimulated acrosome reaction
Na ⁺	Sodium
NNPR	Non-nuclear progesterone receptor
Pb ²⁺	Lead
PCR	Polymerase chain reaction
P-FITC-BSA	Fluorescein conjugated progesterone-carboxymethyloxime-bovine serum albumin
PSAR	Progesterone stimulated acrosome reaction
RACE	Rapid amplification of cDNA ends
RCK3	rat neural cortex voltage-dependent K ⁺ channel
RITC-PSA	Rhodamine-labeled Pisum sativum agglutinin
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
RVGKC	rat voltage gated K ⁺ channel
SEM	Standard error of mean
S4	Transmembrane domain 4 of ion channels
S6	Transmembrane domain 6 of ion channels
TEA	Tetraethylammonium chloride
TMB	3,3',5,5'-tetramethylbenzidine substrate
UV	Ultraviolet
VDCC	Voltage dependent calcium channel

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Abstract:

The objective of our lab is to identify biomarkers which predict fertilization outcome in IVF. Conventional markers such as motility, viability or morphology failed to correlate with fertility potential. Our recent prospective study on infertile couples undergoing their first cycle of IVF demonstrated that >40% of these males exhibited high levels of Pb^{2+} in their seminal plasma. These high levels of Pb^{2+} inversely affected sperm function (i.e. increased spontaneous acrosome loss and decreased ability to undergo an acrosome reaction induced by exposure to progesterone (PSAR) or zona ligands (i.e. mannose). The PSAR was the parameter mostly affected by Pb^{2+} . Our prior preliminary studies on K^{+} channel inhibitors suggested that the effects of progesterone on the human sperm acrosome reaction are mediated by a K^{+} channel. Thus, we hypothesized that sensitivity to Pb^{2+} exposure alters human sperm acrosome reaction by the interaction with a K^{+} channel. To test this hypothesis, we characterized the human sperm K^{+} channel. [1] We examined the ability of human sperm to undergo PSAR in the presence of specific K^{+} channel inhibitors. Our data clearly showed that sperm from men who respond to progesterone exhibited an average of 30-40% inhibition of the PSAR in the presence of K^{+} channel inhibitors. These inhibitors similarly affected spontaneous acrosome reaction (SAR). Interestingly, Pb^{2+} -induced SAR was inhibited by these channel blockers. [2] We localized human sperm surface K^{+} channel binding sites using biotin-coupled CBTX, a specific K^{+} channel blocker. Independent to our observation, other members in the lab showed that Pb^{2+} entry co-localized with K^{+} channel binding sites on human sperm. Thus, biochemical analyses confirmed our prior preliminary findings that a K^{+} channel mediated the effects of progesterone on the human sperm acrosome reaction. Thus, Pb^{2+} associated alterations in human sperm function could be in part mediated by a K^{+} channel. [3] We examined by RT-PCR the expression of KCNA3 in human sperm. For these analyses, we have designed primers from the S4 and S6 region of KCNA3 that contains the ion conduction pore of the channel and binding sites for all K^{+} channel inhibitors used in the biochemical analyses described above. RT-PCR analysis on human sperm using these primers generated specific PCR products that were 98% homologous to KCNA3. Our data suggest that the presence of these K^{+} channel mRNA positively correlated with the ability of human sperm to mount a PSAR. We now identified two individuals whose sperm fail to express the K^{+} channel transcript and do not undergo PSAR. [4] Last, we examined by RT-in situ PCR analyses, the expression pattern of these K^{+} channel transcripts in human sperm. These channel transcripts were expressed over the entire human sperm head in 20-30% of sperm. Thus, these data provide strong evidence for the presence of either KCNA3 or KCNA3 related K^{+} channels in human sperm.

Our prior studies on the rat testis K^{+} channel suggest that more than one isoform of K^{+} channel is expressed in rat testis. Our preliminary analyses on human sperm also suggest the presence of different isoforms of K^{+} channels.

Significant Findings:

- [1] Human sperm acrosome reaction induced by progesterone is markedly inhibited by K⁺ channel inhibitors, TEA (5 mM), CBTX (50 nM) and 4AP (2 mM), indicating that PSAR is mediated, at least in part, by activation of a sperm-associated K⁺ channel.
- [2] Differential response of these inhibitors to PSAR in sperm from different men demonstrates heterogeneity in human sperm K⁺ channel structure/function.
- [3] Charybdotoxin (CBTX) significantly reduced the increase in spontaneous acrosome reaction (SAR) normally produced by Pb²⁺, providing evidence that Pb²⁺ is acting via the sperm K⁺ channel.
- [4] Similar to PSAR, SAR is also inhibited by K⁺ channel inhibitors suggesting that the same K⁺ channel would be involved in the regulation of the SAR.
- [5] Human sperm surface K⁺ channel is localized to the human sperm head. Approximately 38% of sperm exhibited sperm head specific CBTX binding. The same percentages of sperm exhibited non-nuclear progesterone receptor expression (NNPR) (23) suggesting that the sperm surface K⁺ channel could be directly linked to NNPR.
- [6] Characterization of K⁺ channel mRNA from human sperm exhibited the presence of KCNA3 related potassium channel in human sperm. Sperm from 2 men who do not AR in the presence of progesterone failed to express KCNA3 channel transcripts. The presence of these channel transcripts correlated with the ability of human sperm to mount a PSAR.
- [7] RT in situ PCR demonstrated the presence of KCNA3 related channel transcripts over the entire human sperm head.

Usefulness of Findings:

[1] Pb^{2+} associated increase in spontaneous acrosome reaction is in part regulated by a sperm K^+ channel. The spontaneous (“premature”) acrosome loss induced by Pb^{2+} depletes the percentages of sperm capable of undergoing the agonist (i.e. zona) induced acrosome reaction. Thus, the decreased ability to undergo an agonist induced acrosome reaction in sperm exposed to high levels of Pb^{2+} could be due to the increase of premature acrosome loss. Our data suggest that Pb^{2+} induced premature acrosome loss was inhibited by K^+ channel inhibitor, CBTX. Furthermore, Pb^{2+} entry into human sperm is in part, via the sperm associated K^+ channel. Therefore, the IVF failures associated with high levels of Pb^{2+} could be attributable to altered structure/function of sperm associated K^+ channel.

[2] Heterogeneous response of K^+ channel inhibitors to PSAR in different men suggest the presence of more than one isoform of sperm associated K^+ channel. Previously others (19) and we (3-5) have shown that there is heterogeneity in fertile men for the ability of their sperm to undergo PSAR. This heterogeneity could be due to the presence of different isoform of sperm associated K^+ channel.

[3] Our biochemical and molecular data together provide the rationale for examining the correlation of the presence of different isoform of sperm associated K^+ channel to the ability of their sperm to undergo agonist (i.e. progesterone and zona) induced acrosome reaction.

Scientific Report

We have been prospectively evaluating men from infertile couples undergoing their first cycle of fertilization *in vitro* ("IVF") in order to identify biomarkers which predict fertilization outcome and are not related to sperm count, motility, viability or morphology (1). An unexpected finding was that >40% of these males, who were not exposed to lead (Pb²⁺) in their places of work, exhibited blood and seminal plasma Pb²⁺ levels that were above the permissible limit in men occupationally exposed to Pb²⁺ (2). Both blood and seminal plasma Pb²⁺ levels in these men were inversely correlated with the rate of fertilization of metaphase II oocytes in IVF. The negative effect of increased Pb²⁺ on IVF outcome was attributable to altered sperm function, i.e., increased spontaneous acrosome loss and decreased ability to undergo an acrosome reaction ("AR") induced by exposure of capacitated sperm to progesterone ("PSAR") or model zona ligands containing mannose ("Man-AR"). The PSAR was the parameter most severely affected by Pb²⁺.

Concomitantly, results from *in vitro* modeling studies employing potassium (K⁺) channel inhibitors provided evidence that the effects of progesterone on the human sperm acrosome reaction were mediated by a potassium (K⁺) channel (3-6). This K⁺ channel is upstream of the L-type voltage-dependent calcium (Ca²⁺) channel which regulates the sustained Ca²⁺ influx required for the AR (6). K⁺ channels in somatic tissues can be inhibited by Pb²⁺ (7).

Based on these preliminary findings, we hypothesized that sensitivity to Pb²⁺ exposure alters the human sperm AR by interaction with a K⁺ channel. Therefore, the long-term goal for the funded study was to characterize the K⁺ channel expressed in human sperm. The following four sets of experiments were proposed to achieve this aim:

[1] dose-response studies with specific ion channel blockers with the aim of distinguishing among subtypes of voltage-gated channels (e.g., delayed rectifier channels or Ca²⁺-activated channels);

[2] localization of human sperm surface K⁺ channels using biotin-coupled inhibitors;

[3] reverse transcription-polymerase chain reaction (RT-PCR) using sperm RNA as template and primers based on the human voltage-gated K⁺ channel; and

[4] RT *in situ* PCR to examine the expression pattern of K⁺ channels in human testis and sperm.

The first two sets of studies were performed as scheduled, i.e., during the first year of the award. The final two specific aims were focused on the second year of the grant.

Specific Aim #1: dose-response studies with specific ion channel blockers with the aim of distinguishing among subtypes of voltage-gated channels (e.g., delayed rectifier channels or Ca²⁺-activated channels);

[a] The effect of K⁺ channel inhibitors on PSAR: There are at least three general classes of K⁺ channels: [1] cyclic nucleotide-gated channels, [2] voltage-gated channels, including delayed (outward) rectifiers and Ca²⁺-activated channels, and [3] inward rectifiers, including G-protein gated inward rectifiers, ATP-dependent channels and P_{2X} purinergic receptor (8). The conductance of K⁺ channels from sea urchin sperm suggests that they are related to delayed rectifier or Ca²⁺-activated K⁺ channels (9). Therefore, the effects of three K⁺ channel inhibitors were examined: tetraethylammonium chloride ("TEA"), charybdotoxin ("CBTX") and 4-aminopyridine ("4AP"). CBTX (20 nM) is a potent inhibitor of Ca²⁺-activated K⁺ channels in somatic cells (10-12). CBTX is also reported to inhibit delayed rectifier K⁺ channels (13). TEA (0.1-10 mM) and 4AP (0.03-1 mM) specifically inhibit delayed rectifier K⁺ currents (14). Studies in sea urchin demonstrated that TEA (5 mM) completely abolished the egg jelly-induced AR (15), suggesting that delayed rectifier K⁺ channels participate in acrosome exocytosis.

In our studies, capacitated motile sperm populations were divided into four parts. Aliquots were exposed individually to TEA (5 mM), CBTX (50 nM) and 4AP (2 mM). The other part was not exposed to the inhibitors and served as the control. The endpoint of this study was the percentage of capacitated motile sperm undergoing an AR following exposure to 1 ug/mL progesterone. Acrosome loss was assessed following reaction with rhodamine-labeled *Pisum sativum* agglutinin ("RITC-PSA"), a lectin which binds to the sugars in the acrosome content (16). Sperm having undergone an AR exhibit one of two patterns of RITC-PSA binding: [A] a complete absence of any intense sperm head labeling, or [B] a bright band of labeling in the equatorial region, with no detectable labeling of the anterior sperm head or post-acrosomal regions. Pattern B is the result of staining of remnants of acrosome content and converts to pattern A over time (17). Thus, pattern B RITC-PSA binding identifies those sperm having recently undergone AR. In the present study, the PSAR was assessed as the percentages of sperm exhibiting pattern B RITC-PSA binding.

Our preliminary observation suggested that there is heterogeneity in fertile men for the ability of their sperm to undergo PSAR (3-5). This was consistent with previous findings by Calvo et al. (18-19) that fertile men can be separated into two groups according to the ability of sperm to undergo follicular fluid-induced AR. Progesterone is the AR-inducing substance of follicular fluid (20-21). Therefore, we initially grouped fertile men as "Responders" or "Nonresponders", based on the ability of their sperm to undergo PSAR (Figure 1). A $\geq 10\%$ increment in acrosome loss after progesterone exposure was employed to distinguish Responders from Nonresponders.

Figure 1

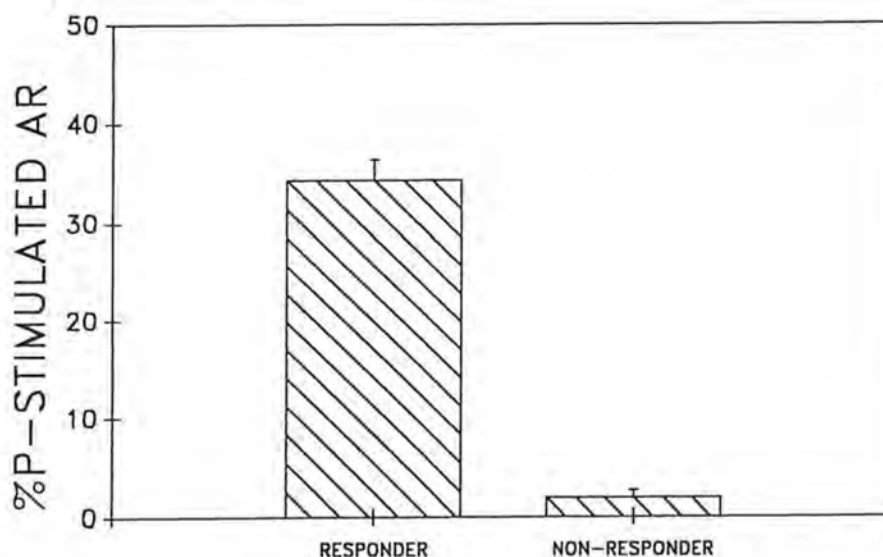


Figure 1: Levels of PSAR were assessed in motile sperm populations from fertile men. The PSAR is calculated as total AR following progesterone exposure minus spontaneous AR divided by total AR after progesterone treatment. Men identified as Responders (n = 37) showed a mean increment in the AR of $34.33\% \pm 2.06$ following progesterone exposure. In contrast, the Nonresponders exhibited only a mean increase in the AR of $1.88\% \pm 0.75$ upon progesterone treatment. PSAR was significantly higher in Responders compared to Nonresponders (Mann-Whitney test, $P < 0.0001$). The bars in the figure represent the mean and standard error of the mean (SEM) for the percentages of sperm undergoing PSAR.

[b] Dose response analysis: To determine the optimum concentration required to detect the effect of K⁺ channel inhibitors on PSAR, capacitated motile sperm populations from fertile Responder donors (mean PSAR = $38.83 \pm 4.20\%$; range, 20.9-49.6%) were divided and exposed to 0.3 mM, 1 mM, 5 mM and 10 mM TEA. Control aliquots were not exposed to the inhibitor. PSAR was then assessed following sperm head labeling with RITC-PSA.

Although inter-male variation in response to TEA was noted in lower levels of TEA (0.3-1 mM), majority of these men exhibited maximal inhibition of PSAR at 5 mM TEA (Figure 2A). These preliminary observations suggested that 5 mM TEA distinguishes between men who respond and do not respond to TEA.

Progesterone activates the sperm K^+ channel by binding to a novel, sperm surface non-nuclear progesterone receptor (NNPR) (22-23). Therefore, to eliminate the possibility that the observed variability in response to TEA was due to reduced expression of NNPR, the percentage of sperm expressing NNPR was quantified following labeling of motile sperm with a cell-impermeant progesterone ligand, fluorescein conjugated progesterone-carboxymethyloxime-BSA (P-FITC-BSA) (Figure 2B).

NNPR are present at all times on the surface of the human sperm head, but are masked by coating factors in the fresh ejaculate (22). During capacitation, these factors are lost and sperm become responsive to exogenous progesterone. Previously we have demonstrated that a threshold value of ≥ 0.3 in the relative increase on incubation in sperm binding P-FITC-BSA in pattern III (see legend to Figure 2B) predicts normal fertilization with a sensitivity of 100%, a specificity of 80%, a positive predictive value of 95.24% and a negative predictive value of 100% (23). With regard to this threshold value, sperm from all 6 men analyzed for response to increasing doses of TEA expressed NNPR above the threshold value suggesting that sperm from these men express the receptor which is directly associated with the K^+ channel. Note that NNPR expression in the one specimen not exhibiting maximal inhibition of PSAR at 5 mM TEA was indistinguishable from the other 5 specimens examined. Thus, the observed inter-male variation in response to TEA was not attributable to reduced NNPR levels. Rather, these data suggest K^+ channel structures differ between men.

Figure 2.

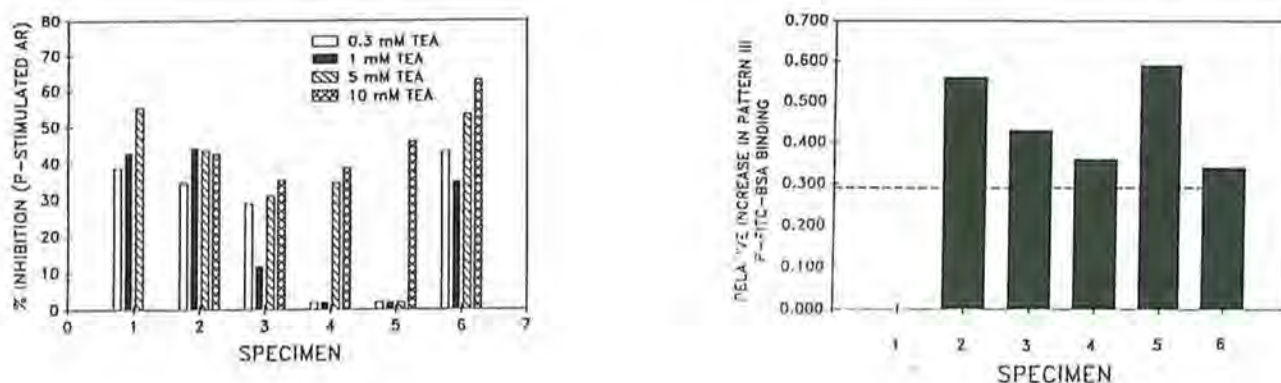


Figure 2. [A] Effect of increasing doses of TEA on PSAR in motile sperm populations from fertile donors ($n=6$). With the exception of specimen #5, 5 mM TEA produces 30-55% inhibition of PSAR. Specimen #5 does not respond to TEA until at the 10 mM dose. There is no significant increase in the inhibition from 5 mM to 10 mM TEA in all other specimens ($P = 0.125$, NS) suggesting that the response of the inhibitor reached plateau level. [B] Duplicate sperm aliquots were analyzed for the percentages of sperm expressing the NNPR to determine whether NNPR expression is normal in these men. Fresh and capacitated sperm were surface labeled with P-FITC-BSA and labeled sperm were examined under UV epi-fluorescence microscopy. Spermatozoa were scored as pattern I, if no P-FITC-BSA binding was observed on the sperm head, as pattern II, if the acrosomal cap was uniformly labeled or as pattern III if only the equatorial and post-acrosomal regions of sperm head were labeled. The conversion of pattern II to pattern III is associated with the AR (22-23). Thus, the relative fraction increase was calculated for each men (i.e., the percentage of sperm binding P-FITC-BSA in pattern III observed for an incubated sample minus the percentage of pattern III P-FITC-BSA binding in the fresh sample divided by the value for the incubated sample). The mean relative increase in pattern III P-FITC-BSA binding from fresh to capacitated sperm was $0.46 \pm 0.05\%$ (0.34-0.56%).

To address this differential sensitivity of K⁺ channel inhibitors among men, the effects of K⁺ channel inhibitors on both spontaneous AR and PSAR was examined in 29 of the Responders analyzed in Figure 1. Results from men who responded to all three inhibitors (n = 19) were analyzed for statistical significance using Wilcoxon signed rank tests (Figure 3).

Figure 3

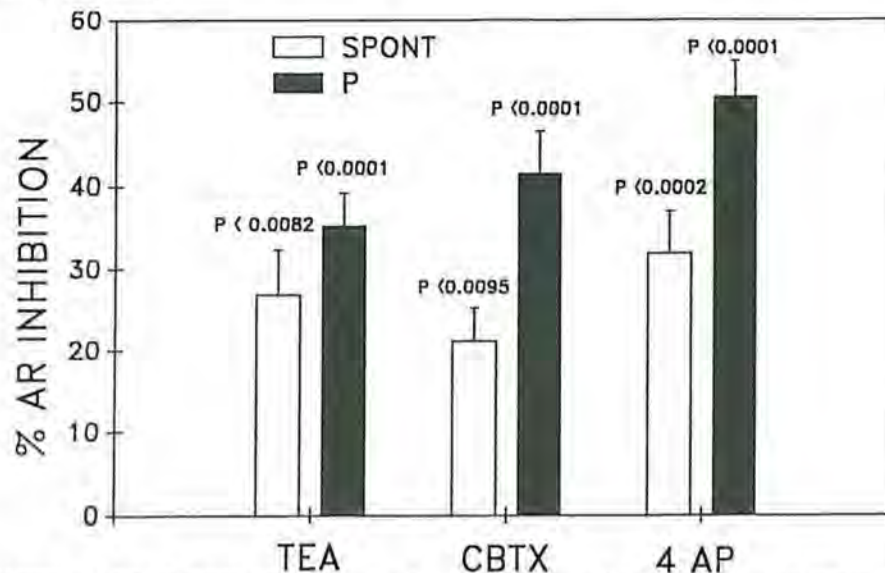


Figure 3: Spontaneous ("SPONT") and PSAR ("P") AR were scored in aliquots treated individually with TEA, CBTX and 4AP and in matched control aliquots from motile sperm from Responder males (n = 19). The bars in the figure represent the mean and standard error of the mean (SEM) for the percentage of AR inhibition resulting from inhibitor exposure.

All three agents markedly inhibited PSAR, providing additional evidence for the expression of delayed rectifier K⁺ channels in spermatozoa. The same agents also significantly inhibited spontaneous AR, albeit at levels lower than for inhibition of PSAR. These data suggest that the same K⁺ channels play a role in the regulation of both the spontaneous and the induced AR.

Our prior studies provided evidence for heterogeneity in sperm K⁺ structure based on differential responses to TEA, CBTX and 4AP, alone or in combinations (5-6). The current study of 37 Responders confirmed these findings (Table 1).

Table 1

	n	TEA	CBTX	4AP
Group I	19	+	+	+
Group II	1	+	-	+
Group III	4	+	-	-
Group IV	1	-	+	-
Group V	2	-	-	-
Group VI	1	-	+	+
Group VII	1	-	-	+

Table 1: The effects of 5 mM TEA, 50 nM CBTX and 2mM 4AP on PSAR were assessed in sperm from Responders; n, the number of individual donors displaying each set of responses; +, PSAR inhibited by $\geq 10\%$; -, no effect on PSAR.

These data demonstrated that, although a majority of men respond to all inhibitors studied, some men respond to one or none of the inhibitors. One possible explanation for this observation is that sperm from different men exhibit inhibition of the PSAR at different concentrations of the inhibitors. TEA, CBTX and 4AP bind to different sites located within the pore of K⁺ channels (24-26). Thus differences in inhibitor responses may be attributable to alterations in the K⁺ channel sequences which are involved in inhibitor binding, i.e., different isoforms resulting from sequence changes in the ion conduction pore (27).

This suggestion is consistent from our findings from the IVF patient study population, where 2/53 men who exhibited high rates of fertilization had seminal plasma Pb²⁺ levels that exceeded 10 ug/dcl (the cutoff for defining the range of blood Pb²⁺ levels in unexposed men) and 3/21 men who exhibited reduced or failed fertilization had Pb²⁺ levels below 10 ug/dcl. We conclude that some factor, which is differentially expressed, controls the effects of Pb²⁺ on sperm function. Structural variations in voltage-dependent Ca²⁺ channel alpha-1 subunits have been associated with the production of sensitivity or resistance to metal ions (28). Since voltage-dependent Ca²⁺ channel and K⁺ channel alpha subunits are related in both sequence and structure (29), it is feasible to suggest that structural variations in the human sperm K⁺ channel alpha subunit could regulate sensitivity to Pb²⁺.

[c] The effect of CBTX on Pb²⁺ exposure: To model the effects of Pb²⁺ on the AR, motile sperm populations from fertile donors (n = 3) were exposed to Pb²⁺ and CBTX alone and in combination (Figure 4; 30). CBTX significantly reduced the increase in spontaneous AR normally induced by Pb²⁺, providing additional evidence that Pb²⁺ is acting via the sperm K⁺ channel.

Figure 4

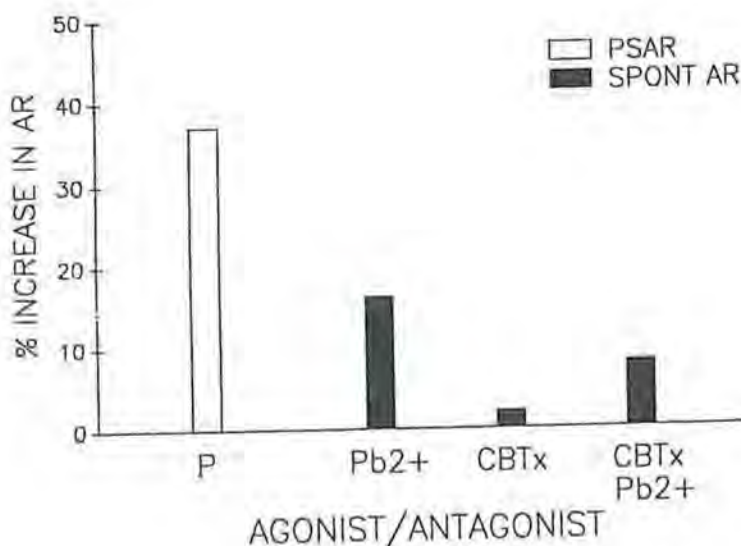


Figure 4: In specimens from men identified by measurements of PSAR ("P") as Responders, Pb²⁺ increased the spontaneous AR ("Pb²⁺") and CBTX ("CBTx") decreased the spontaneous AR. Simultaneous application of Pb²⁺ and CBTX decreased the effect of Pb²⁺ by 50%.

Specific aim #2: localization of human sperm surface K⁺ channels using biotin-coupled inhibitors

The aim of this experimental set was to determine the surface distribution of K⁺ channels on motile human sperm from fertile donors. K⁺ channel-specific antibodies would have been ideal probes for this purpose. However, at the time this study was initiated, such antibodies were not commercially available. Therefore, CBTX was chosen as probe as this scorpion toxin binds to the external surface of K⁺ channels (31).

Sperm from men who exhibited high rates of fertilization in IVF (1, 23; n = 2) were incubated with CBTX and reacted with an anti-CBTX antibody (Alomone Labs, Jerusalem, Israel). The sperm surface distribution of the primary antibody was visualized using FITC-conjugated anti-rabbit IgG preabsorbed with human sperm. Three different conditions were employed in this experiment: [1] anti-rabbit IgG alone, [2] CBTX, anti-CBTX and anti-rabbit IgG, [3] CBTX and anti-rabbit IgG (not shown). The fluorescence patterns of sperm stained with secondary antibody alone, primary and secondary antibody alone, or CBTX plus primary and secondary antibody were similar. We were unable to identify a CBTX-specific labeling pattern by this method. Therefore, labeling of CBTX with biotin was the chosen alternative.

Biotin is a target moiety that can initiate a number of alternative amplification and signal detection schemes. This flexibility allows selection of a scheme which optimizes detection sensitivity while minimizing non-specific signal in each particular application. Another factor influencing the choice of biotin was the commercial availability of reagents with biotin at one end of a 12-24 atom linker arm and moieties reactive with primary amines, sulfhydryls, imidazoles, phenols and carboxylates at the other.

CBTX is a single chain polypeptide of 37 amino acid residues including 1 histidine (imidazole), 1 tyrosine (phenol), 4 lysines (primary amines) and 6 cysteines (sulfhydryl) (32). An ¹H-NMR-based 3-dimensional solution structure (33) and site-specific mutagenesis (32) ruled out cysteines and tyrosine as potential biotinylation targets. Three disulfide linkages involving the 6 cysteines maintained the rigid tertiary structure necessary for functionality. Replacing the one tyrosine with other amino acid residues drastically reduced the mutant CBTX's affinity for K⁺ channel. Histidine and three of the four lysines (excluding Lys 27) were classed as "indifferent residues", at which chemically radical changes did not affect binding affinity (32)). In an accompanying model of a CBTX-K⁺ channel binding complex (32), all four of these residues occupied the upper aqueous-accessible surface, so that attachment of biotin to any of these should not affect K⁺ channel recognition. However because the reagent for biotinylation of histidine, p-diazobenzyl biocytin, reacts with tyrosine under the same conditions, derivatization of charybotoxin's histidine was ruled out. The usual protocol for biotinylation of protein lysines, which uses a large excess of biotinylating agent to maximize biotin incorporation, was obviously unsuitable as it would derivatize all four lysines, including the Lys-27 vital to K⁺ channel binding. Two changes were made with the aim of limiting biotinylation to one or two residues: the molar ratio of biotinylating agent "NHS-LC-Biotin" (Pierce #21335, Rockford, IL) to protein was markedly reduced, and a large excess of Tris buffer (containing tris[hydroxymethyl]aminomethane, a primary amine) was added to stop the reaction after an appropriate time by destroying the remaining NHS-LC-Biotin.

[a] Biotinylation of BSA as control of this experiment to determine levels of biotinylation using reagents from the kit (e.g. HABA): As stated above, one of the primary concerns in biotinylating CBTX was whether biotinylation will reduce or destroy its activity. To prevent this problem, we used BSA as a control as BSA also contains 4 lysine residues.

BSA was incubated with sulfo-NHS-LC-biotin. Different amounts of BSA (0.1-0.4 mg) and different incubation times (1-4 hrs) were tested. The biotinylation reactions were stopped using Tris-glycine buffer. Each reaction was then dialyzed against phosphate buffered saline. The biotin incorporation was determined using [2-(4-hydroxyazobenzene) benzoic acid (HABA) according to the protocols from the manufacturer (Pierce, Rockford, IL). We determined that one mole of biotin per mole of protein was incorporated into BSA under conditions employing 0.15 mg BSA and a 2 hr. incubation.

[b] Biotinylation of CBTX: The protocol developed with a control protein (i.e. BSA) (not shown) was used to biotinylate CBTX. The biotinylated CBTX was subjected to gel filtration chromatography using Bio Gel P polyacrylamide gel. An aliquot of each fraction collected from the column was slot blotted onto Immobilon P membranes (Millipore Corp., Bedford, MA). Membranes were reacted with avidin-horse radish peroxidase (HRP) and developed using a 3,3',5,5'-tetramethylbenzidine substrate (TMB) kit from Vector Laboratories (Burlingame, CA). The individual fractions exhibiting avidin-biotin complexes were stored in aliquots (34).

[c] Labeling rat sperm with biotinylated CBTX: The efficacy of sperm surface labeling with biotinylated CBTX was tested using sperm collected from the cauda epididymis of 4 month old Sprague-Dawley male rats ($n = 2$). After incubation with the probe, rat sperm were reacted sequentially with polyclonal rabbit anti-biotin antibodies, FITC-conjugated anti-rabbit IgG and FITC-conjugated anti-goat IgG (Figure 5; 34).

Figure 5



Figure 5: Paired phase and UV-epifluorescence photomicrographs are shown. Sperm-bound CBTX [a,b] or free biotin [c,d] was detected by indirect immunocytochemistry. Note that free biotin binds only weakly to unfixed sperm and this binding is observed primarily in the post-acrosomal segment of the sperm head and the midpiece [d]. In contrast, CBTX exhibits strong binding over the entire sperm head [b, arrow] and sperm tail.

Examination of stained specimens using UV-epifluorescence optics revealed CBTX bound to the tail of nearly all rat sperm. Based on findings in sea urchin sperm (35), the tail staining could be due to the presence of a hyperpolarization-activated K^+ channel which may be involved in flagellar beating. Approximately 30% of these sperm exhibited additional staining over their heads. We hypothesize that it is the sperm head-associated CBTX binding sites that are involved in the AR.

[d] Labeling human sperm with biotinylated CBTX. Binding of biotinylated CBTX to capacitated motile sperm from fertile donors ($n = 5$) was similarly assessed (Figure 6; 30). Free biotin was used as control. Free biotin bound to the midpiece of all human sperm. In contrast to finding for rat sperm, CBTX did not bind to the human sperm tail. CBTX binding was restricted to the human sperm head. Approximately 38% of sperm exhibited head-specific CBTX binding. The percentages of sperm binding the biotinylated CBTX probe were similar to the percentages exhibiting NNPR expression (i.e; 38%; 23). These data suggest that K^+ channels are expressed on the human sperm head and it could be associated with NNPR.

Figure 6



Figure 6: Paired phase-contrast (A) and UV-epifluorescence (B) photomicrographs of motile human sperm from a fertile donor surface labeled with biotinylated CBTX. Typical results are shown. The arrows in part B point to CBTX binding uniformly over the human sperm head.

Independent to our observation, other members of the laboratory employed autometallography (36-37) techniques to localize the sites of Pb^{2+} entry into sperm (30, 38). These studies demonstrated that Pb^{2+} accumulated uniformly on the human sperm head. The observed distribution of sperm-associated Pb^{2+} co-localized with CBTX binding sites. The same percentages of sperm bound CBTX and bound Pb^{2+} , ~ 38%. These data taken together indicate that a K^{+} channel capable of transporting Pb^{2+} is expressed on the human sperm head.

Specific Aim #3: reverse transcription-polymerase chain reaction (RT-PCR) using sperm RNA as template and primers based on the human voltage-gated K^{+} channel

[a] Characterization of K^{+} channels expressed in rat testis: Our prior experience in cloning the L-type voltage-dependent Ca^{2+} channel α -1C subunit indicated that at least 16 different isoforms can be detected in testis RNA pooled from 20 men (39). Since voltage-dependent Ca^{2+} channel and K^{+} channels α subunits are related (40), it is feasible to suggest that similar variations exist in K^{+} channels. Therefore, pursuing the characterization of K^{+} channel transcripts from human sperm without any additional sequence information would be cumbersome and would markedly increase study costs. As the speed of cloning of the L-type voltage-dependent Ca^{2+} channel α -1 subunits expressed human testis/sperm was significantly increased as result of prior sequence characterization in rat testis, we initially attempted to characterize the full length cDNA of K^{+} channels expressed in rat testis.

A series of paired primers were designed to span the entire cDNA sequence of the rat neural cortex voltage-dependent K^{+} channel cDNA, RCK3: S4F, 5' GTAAGGGTCTTCCGCATCTTCAA; S6R, 5' AACAAATCACAGGAACCGGCAATGC; RVGKC 3F, 5' CATTGCCGGTTCCTGTGAT; RVGKC 3R, 5' AAGAGCTGAGAGAATGCAG; RVGKC 4F, 5' CTGCATTCTCTCAGCTCTT; RVGKC 4R, 5' ATCTTCCTGTGATACTGCA; RVGKC 5F, 5' TGCAGTATCACAGGAAGAT; RVGKC 5R, 5' ATTGAGGAAATCTGAAGGTA; RVGKC 2F, 5' AGGACGTGTTTGAGGCTGCCAA; RVGKC 2R, 5' AACAAATCACAGGAACCGGCAATGC; RVGKC 1F, 5' AGTTCCCTGAGACGCTGCTAG; RVGKC 1R, 5' CATGATATTTCTGGAGAAGGTG; RVGKC 6F, 5' ACCACCTGCTGGAGCCAGAAGC; RVGKC 6R, 5' TGTGTCAGCCTCAAACACGTC. The sequences of the PCR fragments generated using these primers and rat testis cDNA template exhibited 90-95% homology to corresponding regions of RCK3. The longest open reading frame of the cDNA was 1317 bp (18 to 1334 bp). Translation of this open reading frame produced 439 amino acid residues. This amino acid sequence was aligned with RCK3 sequence (Figure 7; 34).

Figure 7

RSKCP	M-----	1
RATKCP	MTVVPGDHLLLEPEAAGGGGDDPPQGGCVSGGGCDRYEPLPPALPAAGEQD	50
RKV3P	MTVVPGDHLLLEPEAAGGGGDDPPQGGCVSGGGCDRYEPLPPALPAAGEQD	50
	*	
RSKCP	-----RYFDPLRNEYFFD	14
RATKCP	CCGERVVINISGLRFETQLKTLQCFPETLLGDPKRRMRYPDPLRNEYFFD	100
RKV3P	CCGERVVINISGLRFETQLKTLQCFPETLLGDPKRRMRYPDPLRNEYFFD	100

RSKCP	RNRPSFDAILYYYQSGGRIRRPVNVPIIDIFSEEIRFYQLGEEAMEKFRED	64
RATKCP	RNRPSLDAILYYYQSGGRIRRPVNVPIIDIFSELRFYQLGEEAMEKFRED	150
RKV3P	RNRPSFDAILYYYQSGGRIRRPVNVPIIDIFSEEIRFYQLGEEAMEKFRED	150

RSKCP	EGFLREEERPLPRDFQRQVWLLFEYPSSGPGARGIAIVSVLVILISIVI	114
RATKCP	EGFLREEERPLPRDFQRQVWLLFEYPSSGPGARGIAIVSVLVILISIVI	200
RKV3P	EGFLREEERPLPRDFQRQVWLLFEYPSSGPGARGIAIVSVLVILISIVI	200

RSKCP	FCLETLPFRDEKDYASPSQDVFEAANNSTSGASSGASSFSDFPFVET	164
RATKCP	FCLETLPFRDEKDYASPSQDVFEAANNSTSGASSGASSFSDFPFVET	250
RKV3P	FCLETLPFRDEKDYASPSQDVFEAANNSTSGASSGASSFSDFPFVET	250

RSKCP	LCIIWFSELLVRFFACPSKATFSRNIMNLIDIVAIIPYFITLGTELAER	214
RATKCP	LCIIWFSELLVRFFACPSKATFSRNIMNLIDIVAIIPYFITLGTELAER	300
RKV3P	LCIIWFSELLVRFFACPSKATFSRNIMNLIDIVAIIPYFITLGTELAER	300

RSKCP	QGNQQAMSLAILRVIRLVRFRIKLSRHSKGLQILGQTLKASRELGL	264
RATKCP	QGNQQAMSLAILRVIRLVRFRIKLSRHSKGLQILGQTLKASRELGL	350
RKV3P	QGNQQAMSLAILRVIRLVRFRIKLSRHSKGLQILGQTLKASRELGL	350

RSKCP	LIFFLFIGVILFSSAVYFAEADDPSSGFNSIPDAFWWAVVTMTTVGYGDM	314
RATKCP	LIFFLFIGVILFSSAVYFAEADDPSSGFNSIPDAFWWAVVTMTTVGYGDM	400
RKV3P	LIFFLFIGVILFSSAVYFAEADDPSSGFNSIPDAFWWAVVTMTTVGYGDM	400

RSKCP	HPVTIGGKIVGSLCAIAGVLTIALPVPVIVSNFNIFYHRETEGEEQAQYM	364
RATKCP	HPVTIGGKIVGSLCAIAGVLTIALPVPVIVSNFNIFYHRETEGEEQAQYM	450
RKV3P	HPVTIGGKIVGSLCAIAGVLTIALPVPVIVSNFNIFYHRETEGEEQAQYM	450

RSKCP	HVGSCQHLSSAEEELRKARSNSTLSKSEYMVIEEGGMNHSAPQTPFKTG	414
RATKCP	HVGSCQHLSSAEEELRKARSNSTLSKSEYMVIEEGGMNHSAPQTPFKTG	500
RKV3P	HVGSCQHLSSAEEELRKARSNSTLSKSEYMVIEEGGMNHSAPQTPFKTG	500

RSKCP	NSTATCTNNPNPNSCVNIKKIFTDV	439
RATKCP	NSTATCTNNPNPNSCVNIKKIFTDV	525
RKV3P	NSTATCTNNPNPNSCVNIKKIFTDV	525

Figure 7: Alignment of the deduced amino acid sequence of the rat testis K⁺ channel ("RSKCP") with RCK3 ("RATKCP") Accession No. M31744) and the related rat KV3 ("RKVP3") K⁺ channel sequences. Perfect sequence conservation is denoted by a "*" below the sequence and conservative amino acid changes are indicated by a ".".

The PCR cloning approach failed to produce the amino terminus of the K⁺ channel from rat testis. To amplify the 5' end of the testis transcript, Rapid Amplification of cDNA Ends (RACE) was utilized. RACE produced a specific product that was resolved on an agarose gel. However, due to the existence of multiple related sequences in the PCR product, automated sequencing failed to generate a specific sequence indicating evidence for isoform diversity (34). We were also unable to amplify the 5' end of the rat testis K⁺ channel using primers derived from rat KV3, (41), which has a 500 bp extension at its 5' end. These data clearly indicated that the 5' end of the rat testis K⁺ channel was distinct from the amino terminus of KV3 or RCK3. Since our goal for this grant proposal was to characterize K⁺ channel in human sperm, we focused our full attention to the human K⁺ channel.

[b] Information gained from amplification of human testis cDNA using RCK3 primers: Our initial analysis with human testis poly(A)⁺ RNA pooled from 20 men and the S4F/S6R primer pair resulted in the generation of a PCR product (Figure 8) which exhibited 85% homology to the rat testis K⁺ channel sequence describe above. The PCR product also exhibited 98% homology with the human voltage-gated K⁺ channel sequence, KCNA3 (Accession No. L23499) (42).

Figure 8



Figure 8: K⁺ channel sequences in human testis cDNA was amplified using primer pairs designed from the rat neural cortex K⁺ channel RCK3: [1] RVGKC1F/1R, [2] RVGKC2F/2R, [3] RVGKC3F/3R, [4] RVGKC4F/4R, [5] RVGKC5F/5R, and [6] S4F/S6R. The H₂O control is shown in lane 7. The sizes of PCR-amplified products were calculated based on their electrophoretic mobility in a 1.2% agarose gel containing ethidium bromide relative to the DNA fragments of the 123 bp molecular weight standard ladder ("M").

The S4F/S6R primer aligned perfectly with the corresponding sequences in KCNA3. All other primers generated from RCK3 exhibited limited alignment with KCNA3 sequences. Therefore, it was not unexpected that only one other primer pair, RVGKC3F/3R, generated specific DNA fragments in sufficient quantity as to permit direct sequence analysis. Note that this PCR exhibited one major product of 357 bp and a minor product of 257 bp. The 357 bp PCR fragment showed significant homology to corresponding region of KCNA3. However, the minor product exhibited strong homology in the first 164 bp to the corresponding KCNA3 sequence and no homology or limited homology for the remainder of the sequence. Interestingly, when the minor PCR product was compared with RCK3 sequences, it exhibited strong homology in the first 131 bp and the last 92 bp to the corresponding region of RCK3. Thus, this minor product exhibited a major internal deletion of 242 bp. These data suggested that more than one isoform of the K⁺ channel is expressed in human testis.

[c] Amplification of K⁺ channel transcripts in RNA populations in sperm: One of the goals of the second year of the grant was to clone the sperm K⁺ channel from human sperm. In preparation for this specific aim, we initially determine the amount of extractable RNA present in human sperm. We reproducibly isolated RNA from human sperm using a commercially available RNA isolation kit designed originally for isolating RNA from body fluids (e.g., blood), PureScript RNA isolation kit (Gentra Systems, Inc.).

Using these reagents, motile human sperm populations isolated by Percoll density gradient centrifugation or swim up are lysed using cell lysis solution provided in the kit and incubated at 55°C for 2 hrs. DNA and proteins are precipitated and removed by centrifugation. The RNA in the supernatant is precipitated with isopropanol. The RNA is the reverse transcribed into cDNA and then assayed for the presence of gene-specific transcripts.

Studies by other members of the laboratory on expression of L-type voltage-dependent Ca²⁺ channel alpha-1C subunits in ejaculated sperm have demonstrated that the amount of Ca²⁺ channel-specific product obtained was proportional to the amount of human sperm cDNA template added to the PCR mixture (43). However, these studies also indicated that <2.8 ug of RNA can be isolated from 4 x 10⁷ sperm. Therefore, all experiments should include primers which amplify transcripts encoding housekeeping genes as controls for RNA recovery.

Based on these findings, RNA isolated from motile sperm populations from fertile donors ($n = 15$) was used as template in RT-PCR with S4F/S6R primer pair. The alpha subunit of a typical voltage-gated K^+ channel consists of a single domain similar to the repetitive domains found in the alpha subunits of Na^+ and Ca^{2+} voltage-gated ion channels (40), which are divided into six putative transmembrane domains designated S1-S6. Transmembrane segment S4 is the voltage sensor. The regions between transmembrane segments S5 and S6 contain the ion conducting pore and binding sites for channel blockers and metals ions. Thus, the S4F/S6R primer pair was designed to amplify a region critical to channel function.

Following RT-PCR using the S4F/S6R primer pair, K^+ channel-specific DNA fragments were identified in 11 of the 15 sperm RNA templates. The assay was repeated two to four times with each template and concordant results were obtained. Typical findings are shown in Figure 9A. To determine whether the inability to amplify a K^+ channel-specific PCR product in the four sperm RNA preparations was simply due to reduced recovery of RNA, duplicate RT reactions were amplified with actin primers. Actin primers produced a PCR fragment of the expected size (500 bp) (Figure 9A, lanes 8-9) and sequence.

Figure 9

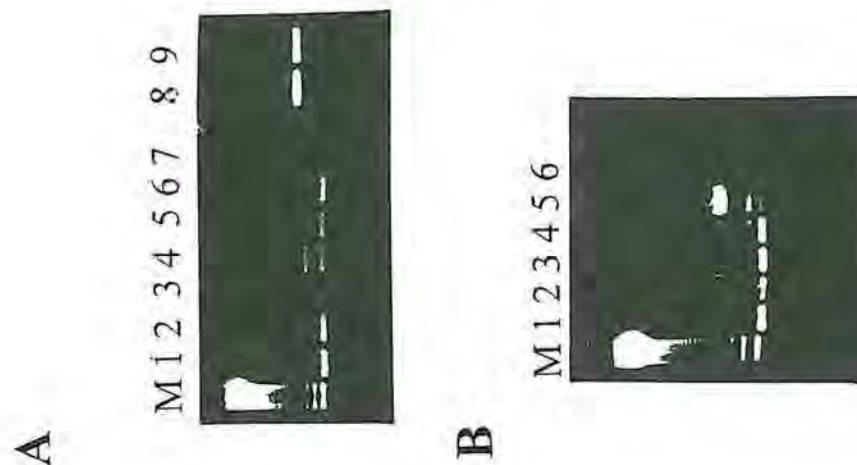


Figure 9: Typical results are shown. The sizes of PCR-amplified products were calculated based on their electrophoretic mobility in a 1.2% agarose gel containing ethidium bromide relative to the DNA fragments of the 123 bp molecular weight standard ladder ("M"). [A] Amplification of sperm cDNA from 8 individual fertile donors with the S4/S6 primer pair resulted in a 316 bp product (e.g., lanes 1-5), whose sequence is identical to that of the PCR product generated from human testis poly(A)⁺ RNA templates. No product was obtained from two specimens (e.g., lane 7). Nevertheless, the ability to amplify actin sequences (lanes 8-9) demonstrated that biologically active RNA was present in these specimens. [B] Sperm cDNA was also amplified using the RVGKC3F/3R primer pair, designed to amplify the 3' end of RCK3. Note that two different sized products were obtained and that each specimen yielded only one product.

Eleven of the sperm populations also exhibited a PSAR which significantly exceeded the normal threshold, e.g., were defined as "Responders" (Figure 10). An examination of the relationship between PSAR and detection of K^+ channel transcripts revealed that gene-specific PCR products were detected in 10 of the 11 Responder specimens and were not found in 3 of the 4 "Non-Responder" specimens. Thus, discordant results were obtained with only two specimens. Note that the observed PSAR for these two discordant specimens was very close to the threshold value (Figure 10). These findings provide further support for the suggested linkage between progesterone binding to NNPR and activation of sperm K^+ channels.

Figure 10

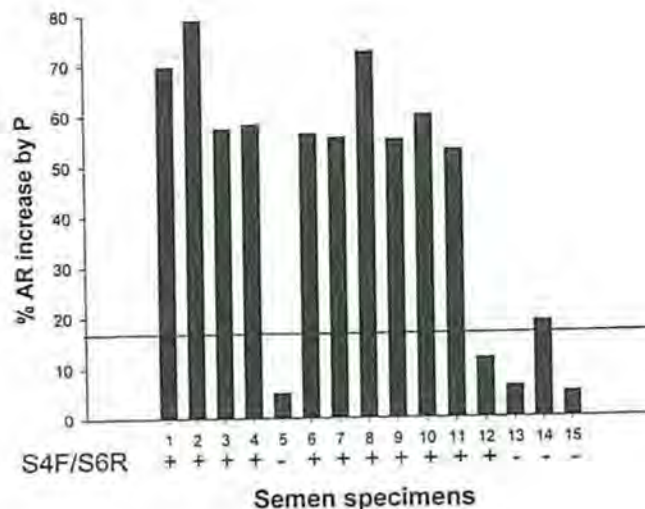


Figure 10: The P-induced increase in acrosome loss for the 15 independent motile sperm populations tested for the presence of K⁺ channel transcripts. The horizontal dashed line represents the threshold for normal response to P treatment. The "+" identifies those specimens which yielded a K⁺ channel gene-specific product following RT-PCR with the S4F/S6R primer pair. The "-" identifies those specimens which failed to produce a K⁺ channel transcript.

The Kappa statistic was used to compare the incidence of agreement between the ability of capacitated sperm to undergo a P-induced increase in acrosome loss at normal threshold levels or higher and the ability to amplify a K⁺ channel-specific product using RNA from ejaculated sperm as template. The simple Kappa coefficient for these 15 specimens was determined to be 0.673 (95% confidence interval = 0.255 to 1.091), indicating good agreement above chance ($P < 0.006$). Thus, expression of KCNA3 K⁺ channel transcripts correlates with a positive acrosome response to P exposure.

The RVGKC3F/3R primer pair was in PCR reactions with cDNA generated from RNA populations from ejaculated human sperm (Figure 9B). Analysis of the DNA fragments indicated the presence of at least two different carboxy termini for the K⁺ channels expressed in sperm. Importantly, only one terminal sequence was expressed in individual RNA isolates. These data support our findings with rat testis cDNA and suggest that K⁺ channel structural variations may underlie the inter-male heterogeneity in the ability to undergo PSAR.

Specific Aim #4: RT in situ PCR to examine the expression pattern of K⁺ channels in human testis and sperm: One of the goals of the second year of the grant was to examine the expression of K⁺ channels in human testis and sperm. Human testis biopsies fixed in Bouins fixative, paraffin embedded, sectioned and collected on in situ Perkin Elmer slides. The sections were deparaffinized, subjected to RT in situ PCR using the S4F/S6R primer pair in conjunction with EZ rTth RNA PCR kit (Perkin Elmer) and digoxigenin-UTP in a Gene Amp in-situ PCR System 1000 (Perkin Elmer) following protocols established in our laboratory (34,44). PCR products were detected using anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) and developed using BCIP/NBT substrate (KPL labs., Inc). Slides were counterstained with nuclear fast red for 15 min and mounted with Permunt. Slides were examined under 400X magnification to localize blue precipitate which is the product of in situ RT-PCR amplification. Seminiferous tubules in these sections were not preserved in order to identify cells that expressed the KCNA3 K⁺ channel transcripts. One of the main problems for this study was that the human testis biopsies were kept in fixative for a long period of time prior to processing. Since fresh human testis biopsies were unavailable, we focused our efforts in attempting to detect KCNA3 K⁺ channel transcript expression in human sperm.

Motile human sperm ($2 \times 10^7/\text{mL}$) isolated using three step percoll density gradient centrifugation were resuspended in 1 X PBS, air dried onto in situ PCR glass slides (Perkin-Elmer), baked at 60°C for 1 hr and fixed in formalin for 16-18 hrs (45) at 4°C . The S4F/S6R primer pair in conjunction with an EZ rTth RNA PCR kit (Perkin Elmer) and bio -16-dUTP (Enzo Diagnostics) was used to amplify KCNA3 RNA sequences in human sperm in a Gene Amp in-situ PCR System 1000. PCR products were detected using 1:2000 dilution of horseradish peroxidase (HRP)-coupled avidin (Bio Rad) in PBS-0.1% Tween-20. Avidin-HRP bound PCR products were developed using a 3,3',5,5'-tetramethylbenzidine substrate (Vector Labs) following manufacturer's protocols. Bio-16-dUTP is substituted for digoxigenin-dUTP in these experiments because avidin-HRP detection system was much stronger than alkaline phosphatase detection method. GAPDHF/GAPDHR primer pairs and HUCH 7F/HUCH 7R designed to amplify a highly conserved region of the L-type voltage dependent calcium channel $\alpha 1\text{C}$ subunit (43) were used in parallel control reactions. Prior to amplification, samples were treated with 10mg/ml pepsin for 1hr and subsequently DNase-treated for 16-18 hrs. Slides were mounted with Permount (Fisher Scientific), examined at X600 magnification with an Olympus BX50 microscope and photographed (Figure 11). PCR products were identified by a positive color reaction (i.e. purple precipitate) with HRP conjugated avidin. Control samples were pepsin-digested for 1 hr and not treated with DNase. Following in situ RT-PCR with S4F/S6R primers, such samples exhibited a high intensity purple precipitate over the entire sperm head suggesting that amplification of genomic KCNA3 as well as cDNA sequences had occurred. In contrast, in samples that were pre-treated with protease and DNase, the PCR signal was markedly reduced and was present over the entire sperm head. The percentages of sperm exhibiting the PCR signal was determined in sperm aliquots from four semen donors. A mean of $24.25 \pm 4.48\%$ was derived. In situ RT-PCR was performed with HUCH 7F/HUCH 7R to examine the expression pattern of voltage dependent calcium channel $\alpha 1\text{C}$ subunit in human sperm. In contrast to KCNA3, VDCC transcripts were localized at the post acrosomal region of human sperm head. A $67.0 \pm 4.73\%$ mean percentage of sperm exhibited PCR signal from three semen donors. In situ RT-PCR with human sperm and GAPDH primers was employed as positive control for this study. GAPDH transcripts were present over the entire sperm head in nearly all sperm. These data provide strong evidence for the expression of KCNA3 and VDCC $\alpha 1\text{C}$ subunit in human sperm.

Figure 11.

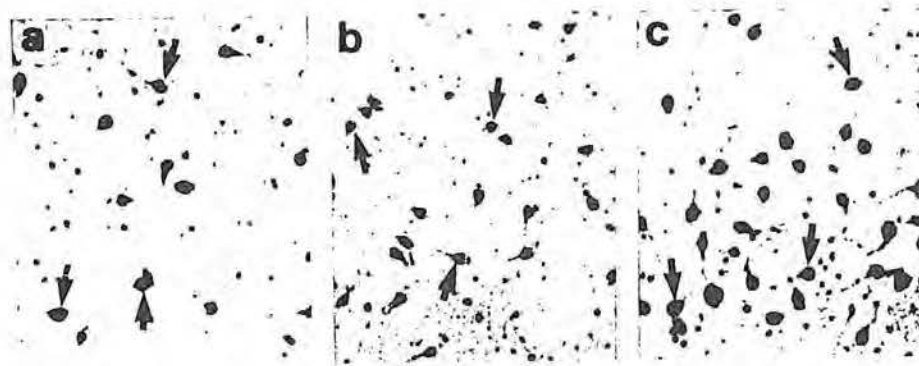


Figure 11. K^+ channel transcripts are observed in human sperm head. Typical results are shown. (A) Following in-situ reverse transcription-polymerase chain reaction (RT-PCR) using S4F/S6R primer pair, a purple precipitate indicative of interaction of horse radish peroxidase-conjugated avidin with biotin-conjugated nucleotides in the PCR product, is observed in ~25% of human sperm over the entire sperm head. Arrows indicate products amplified in mature human spermatozoa. (B) In contrast

to K⁺ channel transcripts, human VDCC $\alpha 1C$ transcripts are restricted to the post-acrosomal region of human sperm and present in ~70% of human sperm. (C) GAPDH transcripts are present over the entire sperm head and present in nearly all sperm.

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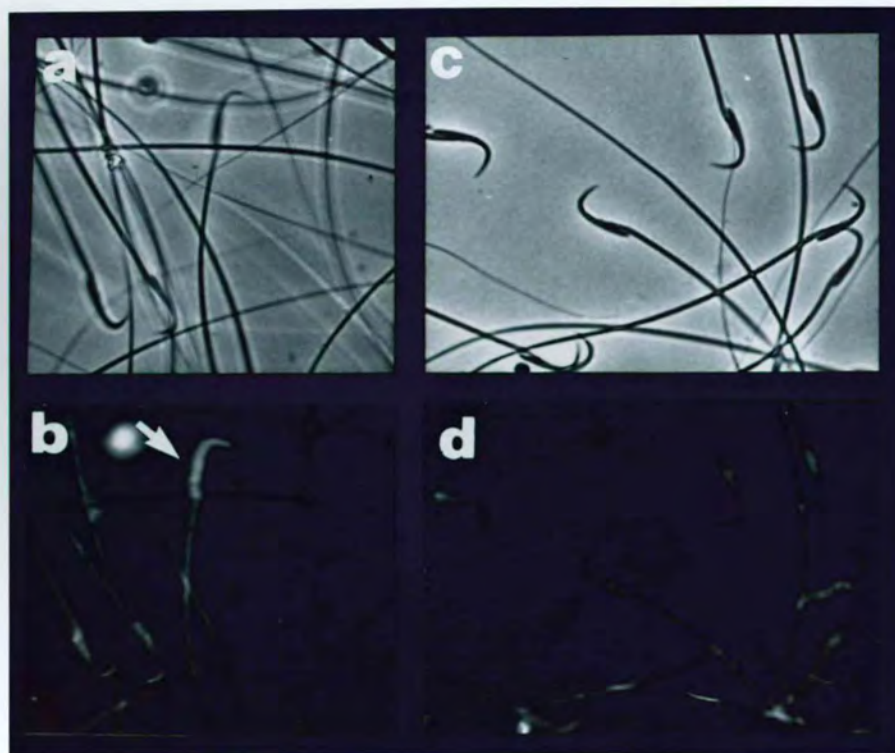
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Appendices

- [1] Jacob A, Hurley IR, Goodwin LO, Cooper GW, Benoff S. Molecular characterization of a voltage-gated potassium channel expressed in rat testis. *Mol Hum Reprod* 2000; 6:303-313.
- [2] Benoff S, Jacob A, Hurley IR. Male infertility and environmental exposure to lead and cadmium. *Hum Reprod Update* 2000;6:107-121.

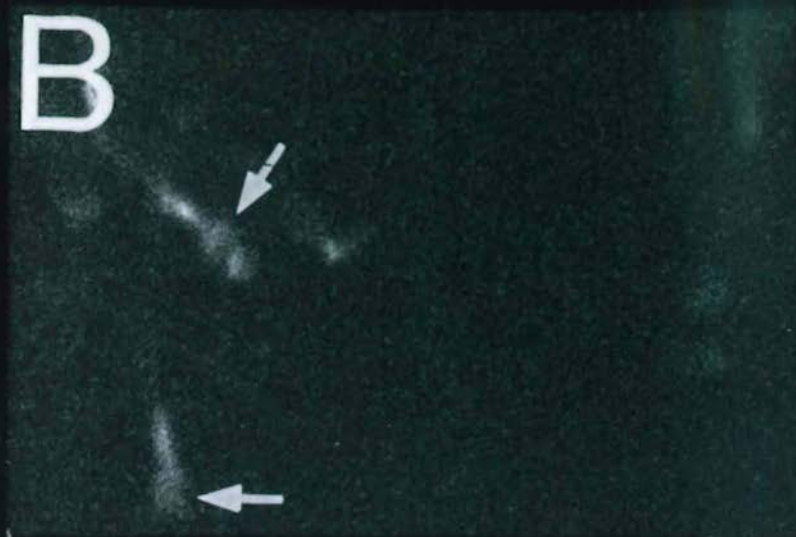
Publications: present and anticipated future.

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- [4] Jacob A, Hurley IR, Cooper GW, Centola GM, Napolitano B, Benoff S. The presence of Kv1.3 mRNA in ejaculated human sperm predicts the ability of sperm to undergo a progesterone-stimulated acrosome reaction – in preparation.
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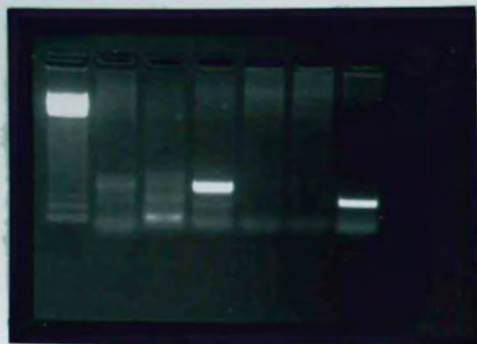




B



M 1 2 3 4 5 6 7



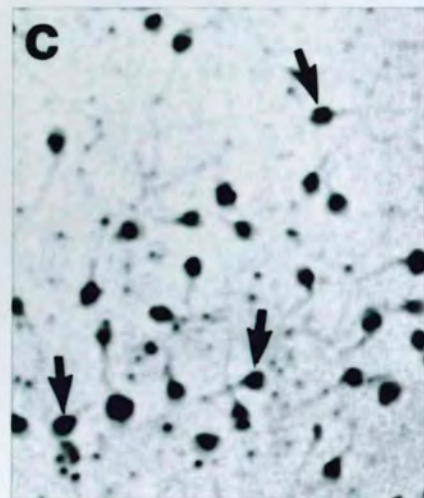
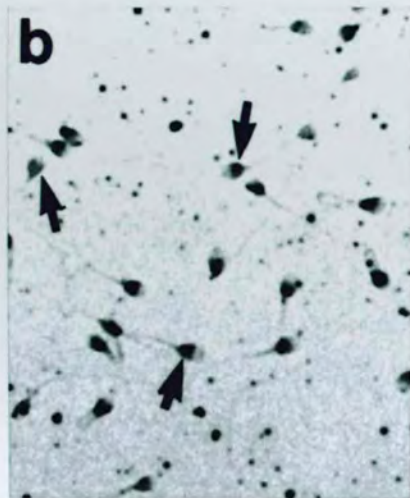
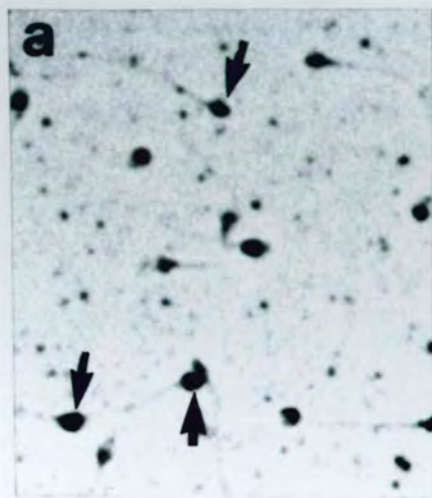


Figure 9

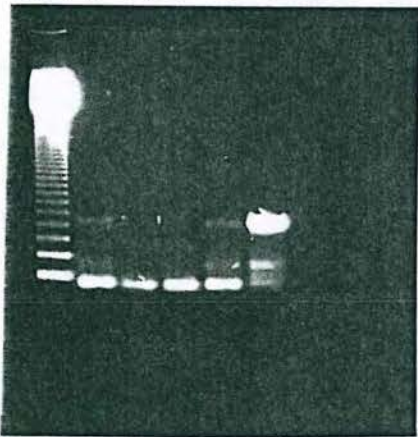
A

M 1 2 3 4 5 6 7 8 9



B

M 1 2 3 4 5 6



Molecular characterization of a voltage-gated potassium channel expressed in rat testis*

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Potassium (K⁺) channels are present in both mammalian testis and spermatozoa. Immunofluorescent detection of sperm-bound biotinylated charybdotoxin, an inhibitor of Ca²⁺-activated and of delayed rectifier K⁺ channels, indicated that these ion channels are uniformly distributed over the surface of both heads and tails of unfixed rat epididymal spermatozoa. Reverse transcription–polymerase chain reaction (RT–PCR) analysis on rat testis RNA with PCR primers, based on known nucleotide sequences of different classes of K⁺ channels, amplified sequences homologous to delayed rectifier K⁺ channels. In-situ RT–PCR on rat testis sections showed that these K⁺ channel transcripts are present in the cytoplasm of primary spermatocytes and post-meiotic elongating spermatids. Northern blot analysis of various rat tissues identified multiple K⁺ channel transcripts, some of which were observed only in testis. An attempt to obtain a full length rat testis K⁺ channel cDNA sequence gave an assembled sequence of 2693 base pairs with >90% homology to a delayed rectifier K⁺ channel, Kv1.3. A method for rapid amplification of cDNA ends was employed to amplify the 5' sequences of the rat testis cDNA but a unique sequence could not be obtained. DNA sequencer traces suggest that multiple related K⁺ channels which differed at their 5' ends were amplified in rat testis.

Key words: charybdotoxin labelling/potassium channel/sperm membrane/spermatozoa/testis

Introduction

The acrosome reaction is an exocytotic event induced by sperm binding to the zona pellucida and requires extracellular calcium ions (Ca²⁺). The elevation of sperm intracellular Ca²⁺ triggers the fusion of plasma and outer acrosomal membranes and release of proteolytic enzymes. This Ca²⁺ enters spermatozoa as the end result of a complex of partially characterized signal transduction pathways activated both by sperm capacitation and by zona binding (review: Benoff, 1998a, 1999).

The acrosome reaction was first identified in echinoderms (Dan, 1956) and has been best studied in these invertebrates. Egg jelly-induced Ca²⁺ influx is accompanied by sodium (Na⁺) uptake and potassium (K⁺) and proton (H⁺) efflux (Schackmann *et al.*, 1978; Schackmann and Shapiro, 1981). Inhibitors of Ca²⁺ and K⁺ channels in somatic cells inhibit induction of the acrosome reaction (Schackmann and Shapiro, 1981) and bind with high affinity to sperm membranes (Kazazoglou *et al.*, 1985; Mikami-Takaei and Yasumasu, 1989), suggesting that sea urchin sperm plasma membrane permeability to Ca²⁺ and K⁺ is regulated by ion channels. Electrophysiological studies of sperm membrane proteins transferred to planar lipid bilayers provided additional evidence for the existence of sperm Ca²⁺ channels (e.g. Guerrero and

Darszon, 1989) and K⁺ channels (e.g. Cook and Babcock, 1993).

While studies in mammalian spermatozoa have been limited, a body of data now indicates that ion currents similar to those observed in sea urchin are associated with the mammalian sperm acrosome reaction and that these ion movements are regulated by monovalent- and divalent-selective cation channels inserted in the plasma membrane (reviewed by Darszon *et al.*, 1999). Recent electrophysiological characterization of the ion channels in human sperm plasma membranes provides evidence for Ca²⁺, K⁺, Na⁺ and chloride (Cl[−]) channels (Chan *et al.*, 1997; Shi and Ma, 1998). Monovalent ion movements precede Ca²⁺ influx (e.g. O'Toole *et al.*, 1996). Sustained Ca²⁺ influx occurs via voltage-dependent Ca²⁺ channels (Florman, 1994; Benoff, 1998b). The exact nature of these Ca²⁺ channels remains controversial, i.e. whether they are low voltage- or high voltage-activated.

Rat and human spermatozoa express L-type voltage-dependent Ca²⁺ channels (Goodwin *et al.*, 1997). Such channels require a large depolarization of membrane potential for full activation (Florman *et al.*, 1992). As in sea urchin spermatozoa (Trimmer *et al.*, 1986), the permeability of mammalian spermatozoa to Ca²⁺ is modulated by alkaline shifts in internal pH (Florman *et al.*, 1989; Zeng *et al.*, 1996). Monovalent cation channels participate in this pH shift (Arnoult *et al.*, 1996). In sea urchin, K⁺ channels are the first to be activated in the pathway leading to the rise in pH of the sperm cytosol

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(Schackmann and Shapiro, 1981; Schackmann *et al.*, 1981; Lee and Garbers, 1986). Thus, K^+ channel activation could be the primary event in the pathway leading to acrosome reaction in mammalian spermatozoa (Benoff, 1999).

There are at least three general classes of K^+ channels: (i) cyclic nucleotide-gated channels, (ii) voltage-gated channels, including delayed (outward) rectifiers and Ca^{2+} -activated channels, and (iii) inward rectifiers, including G-protein-gated inward rectifiers, ATP-dependent channels and P_{2X} purinergic receptors (for review see Christie, 1995). The conductance of K^+ channels from sea urchin spermatozoa suggests that they are related to delayed rectifier or Ca^{2+} -activated K^+ channels (Lievano *et al.*, 1985). Therefore, in this initial attempt to provide a molecular characterization of the K^+ channels in mammalian spermatozoa, we have examined the expression of Ca^{2+} -activated K^+ channels and of delayed rectifier K^+ channels in rat testis.

Materials and methods

Products and reagents

Modified Ham's F-10 medium (Formula No. 90-8050PG) was obtained from Gibco Laboratories (Grand Island, NY, USA). Rat liver cDNA was obtained from Clontech Laboratories (Palo Alto, CA, USA). Unless otherwise specified, all chemicals were reagent grade or higher. Except where noted, all reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Design of a probe to detect sperm K^+ channels

We proceeded on the assumption that voltage-gated K^+ channels, if expressed by rat spermatozoa, would be similar to those detected in sea urchin (Lievano *et al.*, 1985). Charybdotoxin is a potent inhibitor of both Ca^{2+} -activated K^+ channels and delayed rectifier voltage-gated K^+ currents in somatic cells (Deutsch *et al.*, 1991; Brugnara *et al.*, 1993, 1995). Therefore we chose to use charybdotoxin as a probe to localize K^+ channels on the surface membranes of rat spermatozoa. Charybdotoxin is a single chain polypeptide of 37 amino acid residues including one histidine (imidazole), one tyrosine (phenol), four lysines (primary amines) and six cysteines (sulphydryl) (Gimenez-Gallego *et al.*, 1988). A ^1H -NMR-based three-dimensional solution structure (Bontems *et al.*, 1991) and site-specific mutagenesis (Stampe *et al.*, 1994) ruled out cysteines and tyrosine as potential targets for derivatization. Three disulphide linkages involving the six cysteines maintained the rigid tertiary structure necessary for functionality. Replacing the one tyrosine with other amino acid residues drastically reduced the mutant charybdotoxin's affinity for K^+ channel. Histidine and three of the four lysines (excluding Lys-27) were classed as 'indifferent residues', at which chemically radical changes did not affect binding affinity (Stampe *et al.*, 1994). Thus, these indifferent residues can be candidates for labelling (e.g. with biotin).

Biotin is a target moiety that can initiate a number of alternative amplification and signal detection schemes. This flexibility allows selection of a scheme which optimizes detection sensitivity while minimizing non-specific signal in each particular application. Another factor influencing the choice of biotin was the commercial availability of reagents with biotin at one end of a 12–24 atom linker arm and moieties reactive with primary amines, sulphydryls, imidazoles, phenols and carboxylates at the other. In an accompanying model of a charybdotoxin- K^+ channel binding complex (Stampe *et al.*, 1994), all four of these residues occupied the upper aqueous-accessible

surface, so that attachment of biotin to any of these should not affect K^+ channel recognition. However, because the reagent for biotinylation of histidine, *p*-diazobenzyl biocytin, reacts with tyrosine under the same conditions, derivatization of charybdotoxin's histidine was ruled out. The usual protocol for biotinylation of protein lysines, which uses a large excess of biotinylating agent to maximize biotin incorporation, was obviously unsuitable as it would derivatize all four lysines, including the Lys-27 vital to K^+ channel binding. Two changes were made with the aim of limiting biotinylation to one or two residues: the molar ratio of biotinylating agent 'NHS-LC-biotin' (Pierce #21335, Rockford, IL, USA) to protein was markedly reduced, and a large excess of Tris buffer (containing tris[hydroxymethyl]aminomethane, a primary amine) was added to stop the reaction after an appropriate time by destroying the remaining NHS-LC-biotin.

Preparation of biotinylated charybdotoxin

Charybdotoxin (100 μ g) was incubated with 1.5 μ l of 10 mg/ml sulho-NHS-LC-biotin in 100 μ l for 2 h in an ice water bath. At the end of incubation, the biotinylation reaction was stopped by addition of 50 μ l of a buffer containing 0.125 mol/l Tris base, 0.96 mol/l glycine and 10% methanol. Unreacted biotin and Tris-glycine were removed from the reaction mix by gel filtration chromatography over a 1.1 ml column of Bio Gel P polyacrylamide gel extra fine grade (Bio Rad Laboratories, Hercules, CA, USA) in phosphate-buffered saline (PBS). Ten μ l of the 100 μ l fractions collected from the Bio Gel P column was diluted with 49 μ l PBS and blotted using a PR 648 Slot Blot Filtration Manifold (Hoefer Scientific Instruments, San Francisco, CA, USA) onto 100% methanol pre-washed Immobilon-PDVF membranes (Millipore Corp., Bedford, MA, USA). Biotinylated mannosylated bovine serum albumin (BSA; 1–4 μ g) (Sigma No. 7924) was used as a positive control. Membranes were washed twice with PBS and blocked in a TTBS buffer (100 mmol/l Tris-HCl, 150 mmol/l NaCl, 1% Tween-20, 1% BSA) for 1 h at room temperature. To evaluate biotin incorporation into charybdotoxin, membranes were incubated with avidin-horseradish peroxidase (HRP) and the HRP signal was developed using 3,3',5,5'-tetramethylbenzidine substrate (TMB) kit according to protocols from the supplier (Vector Labs, Burlingame, CA, USA). Individual fractions that reacted with avidin-HRP were stored in aliquots with 0.01% sodium azide at -70°C .

Labelling rat spermatozoa with charybdotoxin

Spermatozoa collected from the cauda epididymides of 4 month old Sprague-Dawley male rats (>500 g) were divided into two aliquots. One aliquot was employed for immunohistochemistry with anti-Kv1.3 polyclonal antibodies (see below). The second aliquot was washed with Ham's F-10 medium and incubated with a 1:5 dilution of biotinylated charybdotoxin in PBS for 2 h at 37°C in 5% CO_2 in air. Free biotin replaced the biotinylated charybdotoxin in control reactions. After washing in PBS, spermatozoa were incubated with a 1:100 dilution of rabbit anti-biotin antibody (Catalog No. DS-861; Enzo Diagnostics, New York, NY, USA) for 16–18 h at 24°C . The sperm-bound biotinylated charybdotoxin was then detected with 1:160 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin G (Sigma No. F-0511) in PBS/0.05% BSA at 37°C for 45 min followed by 1:16 dilution of FITC-conjugated anti-goat immunoglobulin G (Sigma No. F-7367) in PBS-0.05% BSA at 37°C for 45 min. Additional control reactions were performed with unlabelled spermatozoa and each of the three antibodies alone or in all possible combinations. Labelled spermatozoa were then air-dried onto glass microscope slides and viewed at $\times 600$ magnification with an Olympus BX50 microscope (Olympus Corp., Lake Success, NY, USA) equipped with epifluorescence optics. Labelled spermatozoa

were photographed at $\times 100$ magnification on 35 mm/400 ASA black and white film (Eastman Kodak Co., Rochester, NY, USA).

Labelling of rat spermatozoa with anti-Kv1.3 polyclonal antibodies

Alternatively, freshly isolated epididymal spermatozoa were washed three times with PBS and smeared onto the well of a pre-cleaned heavy Teflon-coated (HTC) microscope slide (#10-1179; Cel-Line Associates, Inc., Newfield, NJ, USA) and allowed to air dry. Spermatozoa were sequentially reacted with anti-Kv1.3 polyclonal antibody prepared in rabbit (1:50 dilution; #APC-002; Alomone Labs, Jerusalem, Israel) and human spermatozoa pre-absorbed, fluorescein-conjugated sheep anti-rabbit IgG (1:160 dilution; Sigma No. F-0511). The labelling reactions employed buffers containing 0.5% Triton X-100 following protocols established in the laboratory (Benoff *et al.*, 1996). Labelled spermatozoa were viewed as described above.

RNA isolation

Total cellular RNA was isolated from tissues from Sprague-Dawley male rats, 6 months of age with an approximate weight range of 300–400 g, using a modified protocol (Chomczynski and Sacchi, 1987) described previously (Goodwin *et al.*, 1997). The RNA concentration was measured by ultraviolet absorbance at 260 nm and the RNA was stored at -70°C until analysis. For some experiments, total RNA (10 μg) was treated with 13 units of RNase-free DNase I (Gibco-BRL, Grand Island, NY, USA) at room temperature for 45 min. The DNase was then heat-inactivated for 15 min at 65°C . DNase-treated RNA preparations were extracted with phenol/chloroform/isoamyl alcohol and ethanol precipitated in the presence of 3 mol/l sodium acetate, pH 5.2. The precipitate was stored at -70°C until use.

Design of K⁺ channel PCR primers

Sequences for voltage-gated K⁺ channels deposited in the National Center for Biotechnology Information (NCBI) database were identified using BLASTN 2.0 program (Altschul *et al.*, 1997) and accession numbers or key words for known Kv (delayed rectifier) channel sequences. The following sequences were identified: (i) mouse brain K⁺ channel protein-1 (Accession No. Y00305); and (ii) rat mRNA for putative K⁺ channel subunit proteins (RCK1 and RCK2, Accession No. X12589; RCK3, Accession No. X16001; RCK4, Accession No. X16002; RCK5, Accession No. X16003). The structure of the alpha subunit of these channels is shown in Figure 1. The amino acid sequences encoding transmembrane segments S1–S4 are highly conserved among all RCK channels and the amino acid sequences encoding transmembrane segments S5–S6 are identical (Stuhmer *et al.*, 1989).

One RCK nucleotide sequence, RCK3 [the rat neural (cerebral) cortex voltage-dependent K⁺ channel], was chosen at random for use in design of PCR primers. Note that RATKV3 (Accession No. M31744) is essentially homologous to RCK3 [differing only at three nucleotides encoding amino acid number 106, resulting in a substitution of phenylalanine in RATKV3 for leucine in RCK3 (Swanson *et al.*, 1990)] and that these sequences have been renamed Kv1.3 (Chandy and Gutman, 1995).

Initially, a single set of PCR primers complementary to the S4 and S6 regions of Kv1.3 (respectively, S4F and S6R) were synthesized on an Applied Biosystems Model 393 DNA synthesizer (Foster City, CA, USA). These primers were used in the original reverse transcription-polymerase chain reaction (RT-PCR), and in the Northern analysis and in-situ RT-PCR hybridizations. To amplify the full length cDNA, additional sets of paired primers were then synthesized. These primers were defined as rat voltage-gated K⁺ channel (RVGKC) forward (F) and reverse (R) primers and are listed as follows: S4F,

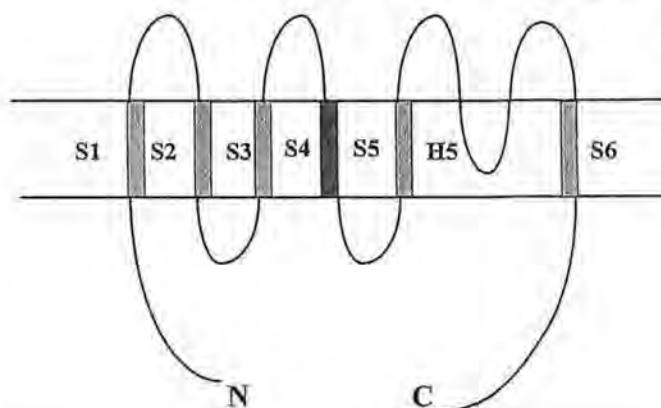


Figure 1. Schematic representation of alpha subunit of a voltage-gated K⁺ channel. The alpha subunit of a typical voltage-gated K⁺ channel consists of a single domain similar to the repetitive domains found in the alpha subunits of Na⁺ and Ca²⁺ voltage-gated ion channels (Catterall, 1995; Benoff, 1998b), which are divided into six putative transmembrane domains designated as S1–S6 (shaded regions). Transmembrane segment S4 is the voltage sensor. The region between transmembrane segments S5 and S6, which protrudes into the membrane and is designated as H5, is the pore region. Amino and carboxy terminals, represented as N and C respectively, extend towards the inside of the cell.

5' GTAAGGGTCTTCCGCATCTTCAA; S6R, 5' AACAAATCACA-GGAACCGGCAATGC; RVGKC1F, 5' AGTTCCTGAGACGCTG-CTAG; RVGKC1R, 5' CATGATATTTCTGGAGAAGGTG; RVGKC2F, 5' AGGACGTGTTTGAGGCTGCCAA; RVGKC2R, 5' AACAAATCACAGGAACCGGCAATGC; RVGKC3F, 5' CATTGC-CGGTTCCTGTGAT; RVGKC3R, 5' AAGAGCTGAGAGAATGC-AG; RVGKC4F, 5' CTGCATTCTCTCAGCTCTT; RVGKC4R, 5' ATCTTCCTGTGATACTGCA; RVGKC5F, 5' TGCAGTATCACA-GGAAGAT; RVGKC5R, 5' ATTGAGGAAATCTGAAGGTA; RVGKC6F, 5' ACCACCTGCTGGAGCCAGAAGC; RVGKC6R, 5' TGTTGGCAGCCTCAAACACGTC.

Based on an earlier study (Kohler *et al.*, 1996), *Rattus norvegicus* Ca²⁺-activated K⁺ channel sequences were retrieved from the NCBI database: (i) rSk1 mRNA (Accession No. U69885); (ii) rSk2 mRNA (Accession No. U69882); and (iii) rSk3 mRNA (Accession No. U69884). As above, one sequence (rSk1) was chosen at random for use in design of PCR primers. Two sets of paired primers were synthesized and are listed as follows: RCAKC1F, 5' TGCTGTTGGT-CTTCAGCAT; RCAKC1R, 5' ACATCCAGGCGGCTCTCCA; RCAKC2F, 5' TAAACGACTCAGTGACTAT; RCAKC2R, 5' AGCCAGCAGATAGAGGCGCT.

Generation of PCR products from rat tissue RNA

To prepare first strand cDNA, total RNA (2 μg) was reverse transcribed in a total volume of 20 μl containing 5 mmol/l MgCl₂, 1 $\times$ reverse transcription buffer (10 mmol/l Tris-HCl, 50 mmol/l KCl, and 0.1% Triton X-100), 1 mmol/l each dNTP, 20 IU of rNasin ribonuclease inhibitor and 20 units of avian myeloblastosis virus (AMV) reverse transcriptase and 0.5 μg of oligo (dT)₁₂ primer using a Reverse Transcription System Kit (Promega, Madison, WI, USA). The reaction was incubated at 42°C for 1 h. The resulting cDNA was precipitated with 3.5 mol/l ammonium acetate and 3 volumes of ethanol at -70°C overnight. The precipitate was centrifuged at 17 000 g for 30 min and the dried pellet was resuspended in 40 μl of H₂O. For PCR amplification of K⁺ channel sequences, 3 μl cDNA, 1 $\times$ PCR buffer (50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.3), 1.5 mmol/l MgCl₂, 250 mmol/l dNTP, 50 pmol of each primer and 2.5 units of Taq

polymerase (Boehringer–Mannheim, Indianapolis, IN, USA) in a final volume of 100 µl were subjected to 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 4 min in a DNA Thermal cycler (Perkin–Elmer, Foster City, CA, USA). The size of the PCR products was estimated by co-electrophoresis of 20 µl of the completed PCR reaction and molecular weight size standards (Bio Rad Laboratories, Hercules, CA, USA) on a 1.2% agarose gel. Size-separated nucleic acids were visualized following ethidium bromide (EB) staining and photographed using a Gel Doc 1000 (Bio Rad).

5' rapid amplification of cDNA ends (RACE)

To amplify the 5' region of the rat testis Kv channels, additional sets of primers were synthesized from Kv1.3 sequences: RVGKCE1R, 5' CCATAGCCTCCTCACCCAGTTGGT; RVGKCE2R, 5' TCAAAACACGTCCTGCGACGGAGAGG; RVGKCE3R, 5' CCTCGGAGAA-GATGTCGATGGGCAC; RVGKCE4R, 5' CCAGCACTGACACGATGGCAATGCC.

Marathon Ready rat testis cDNA (Clontech, Palo Alto, CA, USA) was amplified with 5' adaptor primer (AP1; component of the kit) and a 3' gene-specific primer derived from the Kv1.3 nucleotide sequence (RVGKCE1R or RVGKCE2R) using the Advantage cDNA PCR kit (Clontech) according to manufacturer's protocols. A 1 µl aliquot of this PCR product was used as template in a second PCR reaction using 5' nested adaptor primer (AP2; component of the kit) and nested 3' gene-specific primers (RVGKCE3R or RVGKCE4R) according to methods described in the manufacturer's manual for 'touchdown PCR'. Touchdown PCR (Don *et al.*, 1991; Roux, 1995) involves using an annealing temperature during the initial PCR cycles that is several degrees higher than the T_m of the adaptor primer ($T_m > 70^\circ\text{C}$). This allows the gene-specific product to accumulate. Then the annealing temperature is reduced to the adaptor primer T_m (68°C).

DNA sequence analysis

Amplified products were directly purified with Wizard PCR kit (Promega). The purified fragments were then sequenced directly using the original primers in an automated Model 370 DNA Sequencing System (Applied Biosystems, Foster City, CA, USA) following manufacturer's protocol for fluorescence-based DNA sequencing with Taq polymerase. The sequences obtained were assembled to construct a full length cDNA sequence. Compiled sequences were translated by PC/Gene Release 7.1 (IntelliGenetics, Mountain View, CA, USA) and the longest open reading frame was identified. Derived amino acid sequences were then aligned with the Kv1.3 amino acid sequence and with the sequences deposited in the NCBI database using BLASTN 2.0 program.

Northern blot analysis

Total RNA (10 µg) from various rat tissues was size-fractionated on denaturing agarose gels, transferred to nylon membranes (Micon Separation Inc., Westboro, MA, USA) and hybridized according to published protocols (Maniatis *et al.*, 1987) with ^{32}P -random-primed probe derived from a 2169 nucleotide clone encoding a portion of the rat testis-specific L-type voltage-dependent Ca^{2+} channel α -1C subunit (Goodwin *et al.*, 1997) or actin cDNA from exons 4 and 5 (Goodwin *et al.*, 1999). The membranes were then exposed to X-ray film (Kodak, New Haven, CT, USA) and the resultant autoradiogram photographed on GS-700 imaging densitometer (Bio Rad). The blot was stripped and reprobed with a ^{32}P -labelled PCR product generated using rat testis cDNA and S4F/S6R primers as per the standard laboratory protocol.

In-situ RT-PCR hybridization

We chose to use cryosections of rat testis rather than sections from paraffin-embedded tissues due to ease of preparation (Ausubel *et al.*,

1991). Rat testis frozen sections (5 µm) were collected on in-situ PCR glass slides (Perkin–Elmer) and fixed in formalin as described earlier (Nuovo, 1994). The S4F/S6R primer pair in conjunction with an EZ rTth RNA PCR kit (Perkin–Elmer) and digoxigenin dUTP (Boehringer–Mannheim) was used to amplify Kv1.3 RNA sequences in these testis sections in a Gene Amp in-situ PCR System 1000 (Perkin–Elmer) following protocols established in the laboratory (Goodwin *et al.*, 1998). Actin primers (forward and reverse, to exon 5) and primers derived against *E. coli* DNA origin of replication (forward primer 5' CGAGATTACAAAAGTTACCTG and reverse primer 5' CGTTAGCCCCACCCAGCAAAA) were used in parallel control reactions. Prior to PCR amplification, testis sections were treated with 2 mg/ml pepsin for either 15 min or 30 min and subsequently DNase-treated as described previously (Goodwin *et al.*, 1998). Testis sections which were protease-treated for 30 min but not DNase-treated were also included in this experiment. PCR products were detected using alkaline phosphatase-coupled anti-digoxigenin antibody (Boehringer–Mannheim) as previously described (Goodwin *et al.*, 1998). Slides were mounted with Permout (Fisher Scientific, Pittsburgh, PA, USA), examined at $\times 600$ magnification with an Olympus BX50 microscope and photographed.

Results

Labelling rat spermatozoa with charybdotoxin

Before embarking upon an extensive molecular search, it was imperative to determine if the surface of mature rat spermatozoa contained K^+ channels related to Ca^{2+} -activated or delayed rectifier K^+ channels, and, if present, simultaneously determine their distribution within the various compartments of the sperm surface membrane. K^+ channel-specific antibodies would have been ideal probes for these purposes. However, at the time this study was initiated, such antibodies were not commercially available. Labelling of charybdotoxin with biotin was the chosen alternative. Charybdotoxin, one of a class of scorpion peptide neurotoxins, blocks voltage-gated K^+ channels by binding specifically to the external surface of K^+ channels at low concentrations (1 pmol/l to 10 nmol/l) (Deutsch *et al.*, 1991; Brugnara *et al.*, 1993, 1995; Stampe *et al.*, 1994). It is implausible that a simple peptide such charybdotoxin would exhibit non-specific binding with such a high affinity to a random site on the sperm surface. Dissociation constants for non-specific interactions are typically in the micromolar range or higher, even at lower ionic strengths. Inhibition constants in the picomolar range, on the other hand, imply a specific binding site, as has been documented for this ion channel inhibitor.

Charybdotoxin was minimally biotinylated to preserve its K^+ channel binding activity (see Materials and methods) and unfixed rat epididymal spermatozoa were incubated with this preparation. Sperm-bound probe was localized following triple antibody staining employing anti-biotin antibodies. Spermatozoa incubated with free biotin served as a control. The pattern of non-specific staining observed with free biotin clearly differed from the staining pattern obtained with biotinylated charybdotoxin. While free biotin weakly reacted with the post-acrosomal segment of the sperm head and with the midpiece region (Figure 2C and D), a strong fluorescence signal was detected over the entire sperm head and sperm tail in aliquots labelled with the biotinylated charybdotoxin (Figure 2A and B).

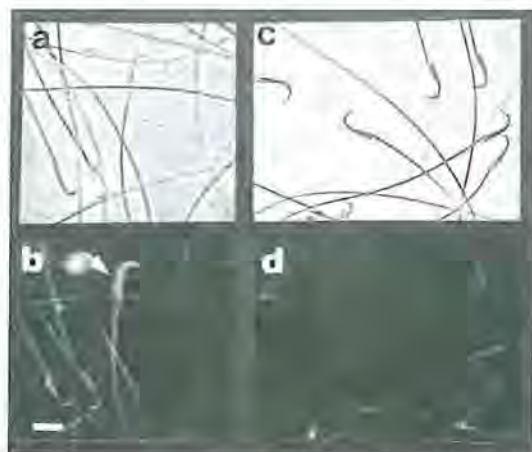


Figure 2. Localization of rat sperm K^+ channels using biotinylated charybdotoxin. Paired phase and epifluorescence photomicrographs are shown. Sperm-bound charybdotoxin (a, b) or free biotin (c, d) was detected by indirect immunocytochemistry (see Materials and methods). Note that free biotin binds only weakly to unfixed spermatozoa and this binding is observed primarily in the post-acrosomal segment of the sperm head and the midpiece (d). In contrast, charybdotoxin exhibits strong binding over the entire sperm head (b, arrow) and sperm tail. Scale bar = 15 μ m.

These studies were subsequently repeated using a polyclonal antibody directed against a recombinant fusion protein containing the carboxy terminus of the human homologue of the rat delayed rectifier K^+ channel Kv1.3. As observed for biotinylated charybdotoxin, this antibody specifically bound over the entire rat sperm head (not shown). Sperm-bound antibody was also detected on tails.

These data provide evidence for the presence of proteins related to voltage-gated K^+ channels in mature rat spermatozoa.

A delayed rectifier K^+ channel is expressed in rat testis

The initial attempt to amplify K^+ channel sequences in RNA isolated from rat testis employed primer pairs RCAKC1F/1R and RCAKC2F/2R, which were designed from a small-conductance, Ca^{2+} -activated K^+ channel expressed in brain (Kohler *et al.*, 1996). PCR products of the expected size, 618 and 420 bp, respectively, were obtained with brain cDNA template. In contrast, PCR reaction performed with rat testis cDNA and the RCAKC1F/1R primer pair yielded a series of fragments unrelated in size to the gene-specific products (not shown). PCR reactions performed with rat testis cDNA and the RCAKC2F/2R primer pair resulted in amplification of 320 and 148 bp DNA fragments (not shown). Direct sequencing of the 320 bp product revealed 64–65% identity to *H.sapiens* mRNA for nuclear proteins SA-1 (EMBL 275331) and SA-2 (EMBL 275330). Direct sequencing of the 148 bp fragment yielded a sequence without significant homology to any sequence deposited in the NCBI database.

To search for the expression of delayed rectifier K^+ channels in rat testis, PCR was performed with primers to the S4–S6 region of Kv1.3. This region contains the ion conducting pore. A 316 bp PCR product was amplified from cDNA generated from testis (Figure 3). A similar-sized product was obtained from rat brain and liver templates (Figure 3). The nucleotide

Voltage-gated K^+ channel in rat testis



Figure 3. Expression of voltage-gated, delayed rectifier K^+ channels in rat tissues. Total RNA isolated and reverse transcribed to cDNA from rat testis (TESTIS), rat brain (BRAIN) and cDNA from rat liver (LIVER) was amplified using S4F/S6R primers. A 316 bp fragment was amplified from RNA from all tissues tested. Fragment size was determined by extrapolation from the 123 bp DNA ladder molecular weight size standard (MW; Cat No. 15613-011; Gibco-BRL, Grand Island, NY, USA). No EB staining is detectable in the lane containing the H_2O control (H_2O).

sequences obtained from direct sequencing of these PCR products were aligned with the sequence of the S4–S6 region of Kv1.3 sequence (not shown). This analysis demonstrated that the PCR products exhibited 96.8% homology with Kv1.3 cDNA. A further sequence homology search using BLASTN 2.0 non-redundant DNA sequence alignment from NCBI (Altschul *et al.*, 1997) demonstrated that the PCR products from rat tissues were also related to rat voltage-gated K^+ channel protein, RATRGK5 (Accession No. M30312), with similar homology in the corresponding region of S4–S6 (not shown).

These data suggest that mRNA for small-conductance, Ca^{2+} -activated K^+ channels are not abundant in rat testis. In addition, these observations provide the first molecular evidence that a gene encoding a voltage-gated, delayed rectifier K^+ channel is expressed in rat testis.

Localization of voltage-gated K^+ channel RNA transcripts in rat testis

In-situ RT-PCR was performed using S4F/S6R primers and digoxigenin dUTP as a tagged nucleotide in order to examine the expression pattern of voltage-gated K^+ channels in rat testis (Figure 4A). PCR products were identified by a positive colour reaction (i.e. blue precipitate) with the alkaline phosphatase-conjugated anti-digoxigenin antibody.

Control testis sections were pepsin-digested for 30 min and not treated with DNase. Following in-situ RT-PCR with the S4F/S6R primer pair, such sections exhibited a relatively uniform, high intensity blue precipitate, suggesting that amplification of genomic K^+ channel sequences had occurred (not shown). In contrast, in sections that were both protease- and DNase-treated, the PCR signal was markedly reduced and limited primarily to the cytoplasm of primary spermatocytes in stages VII through IX/X of rat spermatogenesis. In addition, there was a dramatic decrease in the ability to detect K^+

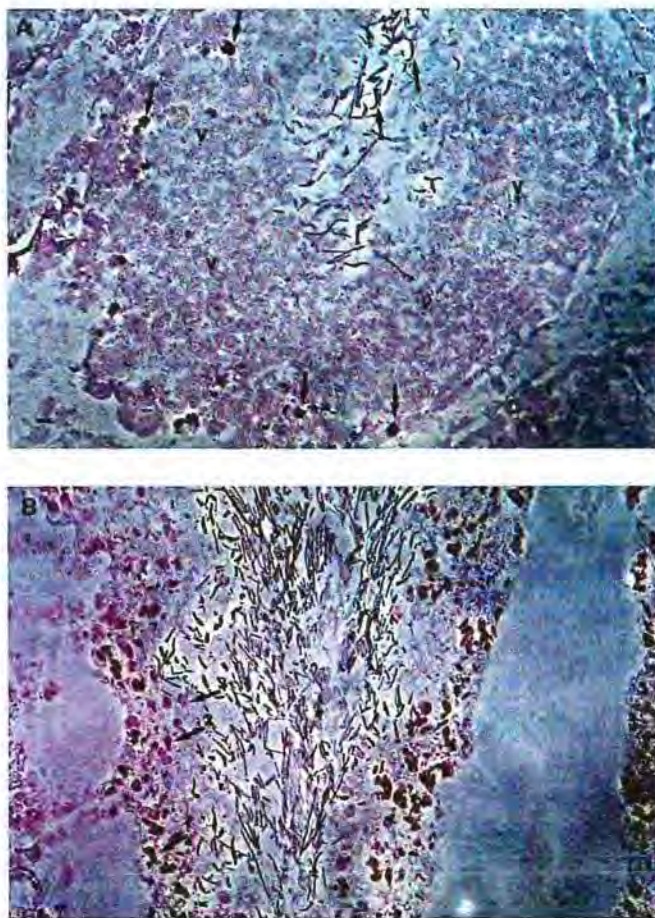


Figure 4. K^+ channel transcripts are observed in post-meiotic male germ cells. Typical results are shown. Scale bars represent 10 μ m. (A) Following in-situ reverse transcription-polymerase chain reaction (RT-PCR) using the S4F/S6R primer pair, a blue precipitate, indicative of interaction of alkaline phosphatase-conjugated antidigoxigenin antibody with digoxigenin-conjugated nucleotides in the PCR product, is observed over the heads of post-meiotic elongating spermatids and mature spermatozoa. Arrows indicate products amplified in mature spermatozoa present in the lumen of seminiferous tubules as well as in primary spermatocytes located in the adluminal compartment and basal cells (which could include spermatogonia and muscle cells). Open arrow heads point to smaller precipitates occurring throughout the seminiferous epithelium, which may represent decaying K^+ channel transcripts or low levels of K^+ channel gene transcription. Further studies are required to distinguish between these two possibilities. (B) In contrast to K^+ channel transcripts, actin transcripts are present throughout the seminiferous tubules. A gradient of precipitate densities is observed from the cells in the basement membrane (i.e. darker; arrow no. 1) to the cells closer to the lumen (i.e. lighter; arrow nos. 2 and 3). Amplified product is also detectable in mature spermatozoa.

channel-specific transcripts when the sections were exposed to shorter protease digestion time (data not shown).

Protease pretreatment can distort the morphology of cryosections (Nuovo, 1994). Therefore sperm cell associations in sectioned rat seminiferous tubules were identified using a staging scheme (Leblond and Clermont, 1952). In these frozen sections, no signal was seen in the regions of the seminiferous tubule where immature spermatozoa were present near the lumen of the tubule. Signal was only seen above the germinal

layer in cells presumed to be primary spermatocytes by their association with mature sperm heads and spermatozoa undergoing release (spermiogenesis) near the tubule lumen (Figure 4A). K^+ channel transcripts were also observed in mature spermatozoa released into the lumen of the seminiferous tubules (Figure 4A). No blue precipitate was observed in negative controls, i.e. testis sections which were not protease digested or testis sections amplified with *E.coli* primers (data not shown), confirming that the signal present in the post-meiotic elongating spermatids and mature spermatozoa was specific for K^+ channel mRNA.

In-situ RT-PCR with rat testis sections and actin primers was employed as positive control for this study. Actin transcripts were detected throughout the testis, including cells in interstitial regions. The density of the actin hybridization signal was more abundant in cells closer to the basement membrane of the seminiferous tubules as compared to cells closer to the lumen, i.e. with differentiation of cells of the male germ line (Figure 4B). This is consistent with reports that actin mRNA levels are higher in pre-meiotic cells than in post-meiotic cells (Hecht and Penshow, 1987; Caldwell and Handel, 1991).

These data illustrate the differing patterns of gene expression which occur during spermatogenesis in the rat.

Northern blot analysis

To provide an estimate of the number of structurally distinct delayed rectifier K^+ channel sequences expressed in different rat tissues, total RNA from various rat tissues were size-fractionated by electrophoresis through denaturing agarose gels, blotted onto nylon membranes and hybridized with ^{32} P-labelled 316 bp PCR product generated using rat testis RNA as template and the S4F/S6R primer pair (Figure 5). A 5 kb RNA transcript was detected in rat heart, thymus, kidney, spleen, skeletal muscle, liver, lung and testis which hybridized to the Kv1.3 cDNA probe sequence. Two less abundant transcripts of 4.2 and 2.4 kb were observed only in the testis.

It is important to note that a hybridization signal was detected only after a relatively long exposure of the membrane to X-ray film, i.e. 10 days, as compared to the 2 day exposures required to detect L-type voltage-dependent Ca^{2+} channel α -1C subunit transcripts and actin transcripts (Goodwin et al., 1999). These data suggest that transcripts encoding K^+ channels in testis are less abundant than those encoding Ca^{2+} channels or a housekeeping gene.

Attempts to amplify full length rat testis K^+ channel cDNA sequences

To further characterize the K^+ channel expressed in rat testis, six sets of paired primers were synthesized. These primers were designed so that they spanned the entire cDNA sequence of the rat neural cortex voltage-gated K^+ channel Kv1.3 (see Materials and methods and Figure 6). These primers were employed in PCR reactions using testis cDNA as template.

With the one exception of the RAKC6F/RAKC6R primer pair, the primer sets amplified robust products of the expected size (not shown). However, a series of additional minor products was observed in each case. Such products could have resulted from amplification of related sequences or non-

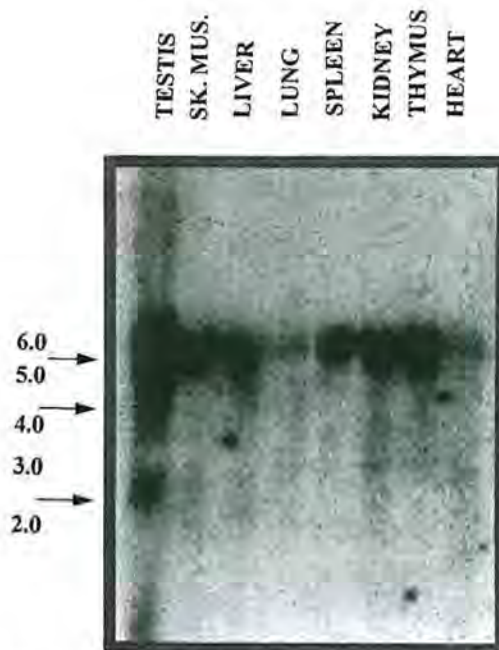


Figure 5. Northern hybridization of total RNA from different rat tissues with ³²P-labelled polymerase chain reaction product prepared with rat testis RNA template and the S4F/S6R primer pair. The numbers to the left of the blot represent sizes (in kb) extrapolated from the 1 kb marker RNA ladder (no. 15620-016; Gibco-BRL). Arrows identify transcripts which hybridized with the probe. A 5 kb transcript was detected in all tissues analysed. Additional 4.2 and 2.4 kb transcripts were detected only in testis. SK, MUS. = skeletal muscle.

M 1 2 3 4 5 6 7



Figure 6. Robust polymerase chain reaction (PCR) products were obtained when total RNA isolated from rat testis was reverse transcribed into cDNA and then amplified using primers designed from internal sequences of the rat cerebral cortex voltage-dependent potassium channel Kv1.3. The region amplified by a specific primer pair is as indicated: (1) S4F/S6R (nucleotides 962 to 1274), (2) RVGKC3F/3R (nucleotides 1276 to 1793), (3) RVGKC4F/4R (nucleotides 1775 to 2392), (4) RVGKC5F/5R (nucleotides 2374 to 2940), (5) RVGKC2F/2R (nucleotides 669 to 1298), and (6) RVGKC1F/1R (nucleotides 228 to 841). In contrast, primers designed to amplify the 5' end of the transcript (7), RVGKC6F/6R (nucleotides 27 to 693), failed to elicit a gene-specific product; only primer dimers were detected. The sizes of PCR-amplified products were calculated based on their mobility in a 1.2% agarose gel containing ethidium bromide relative to the DNA fragments of the 123 bp molecular weight marker ladder (M).

RSKCP	M-----	1
KV1.3	MTVVFGDHLLEPEAAGGGGDPQGGCVSGGCDRYSPPLPALPAAGEQD	50
	*	
RSKCP	-----RYFDPLRNEYFFD	14
KV1.3	CCGERVINISGLRFETQLKTLCPFPETLLGDPKRRMRVFDPLRNEYFFD	100

RSKCP	RNRPSFDAILYQSGGRIRRPVNPIDIFSEBIRFYQLGEAMEKFRD	64
KV1.3	RNRPSLDAILYQSGGRIRRPVNPIDIFSEBIRFYQLGEAMEKFRD	150

RSKCP	EGFLREERPLPRRDFQRQVWLLFEYPSSGPARGIAIVSVLVILISIVI	114
KV1.3	EGFLREERPLPRRDFQRQVWLLFEYPSSGPARGIAIVSVLVILISIVI	200

RSKCP	FCLETLPFRXKDYSPASQDVFEAANNSTSGASSGASSFSDPPFFVET	164
KV1.3	FCLETLPFRDEKDYSPASQDVFEAANNSTSGASSGASSFSDPPFFVET	250

RSKCP	LCIIWFSFELLVRFFACPSKATFSRNIMNLIDIVAIIPYFITLGTALAER	214
KV1.3	LCIIWFSFELLVRFFACPSKATFSRNIMNLIDIVAIIPYFITLGTALAER	300

RSKCP	QNGGQQAMSLAILRVIRLVVRFRIFKLSRHSKGLQILGQTLKASMRGL	264
KV1.3	QNGGQQAMSLAILRVIRLVVRFRIFKLSRHSKGLQILGQTLKASMRGL	350

	b c	
RSKCP	LIFFLFIGVILFSSAVYFAEADDPSSGFNSIPDAFWAVVMTITVGYGDM	314
KV1.3	LIFFLFIGVILFSSAVYFAEADDPSSGFNSIPDAFWAVVMTITVGYGDM	400

RSKCP	HPVTIGGKIVGSLCAIAGVLTIALPVPVIVSNFNFYHRETEGEEQAQYM	364
KV1.3	HPVTIGGKIVGSLCAIAGVLTIALPVPVIVSNFNFYHRETEGEEQAQYM	450

	a	
RSKCP	HVGSCQHLSSAEBLRKARSNSTLSKSEYMVIEEGGMNHSAFPQTPFKTG	414
KV1.3	HVGSCQHLSSAEBLRKARSNSTLSKSEYMVIEEGGMNHSAFPQTPFKTG	500

	d	
RSKCP	NSTATCTTNPNPNSCVNIKKIFTDV	439
KV1.3	NSTATCTTNPNPNSCVNIKKIFTDV	525

Figure 7. Alignment of the deduced amino acid sequence of the rat testis voltage-gated K⁺ channel (RSKCP) with Kv1.3 sequences. Perfect sequence conservation is denoted by an asterisk below the sequence and conservative amino acid changes are indicated by a full point. Lines in the figure denote sites for modification of channel activity: (a) a potential site for tyrosine phosphorylation (Holmes *et al.*, 1996), (b, c) two potential protein kinase C sites, and (d) a potential protein kinase A site (Stuhmer *et al.*, 1989).

functional shortened transcripts or from amplification of contaminating genomic DNA. To eliminate the latter possibility, total RNA from testis was digested with RNase-free, DNase I prior to RT into cDNA. After DNase treatment, only a single PCR product of the predicted size was obtained (Figure 6).

Each PCR product was purified and subjected to direct DNA sequencing. The sequence of each PCR fragment of the testis K⁺ channel sequence exhibited a 90–95% homology with its corresponding sequence within Kv1.3. The sequences from the individual PCR products were assembled into a 2693 base pair sequence. The longest open reading frame in this sequence is 439 amino acids in length. This deduced amino acid sequence was aligned with the amino acid sequences of Kv1.3, demonstrating an overall 99% identity in the corresponding region (Figure 7). Note that a phenylalanine is present at position 106 of the partial amino acid sequence of the rat testis K⁺ channel. The sites for phosphorylation by protein kinase C (PKC) and by tyrosine kinase (TK) which are present in Kv1.3 were also identified in the testis K⁺ channel.

These data demonstrate that a K⁺ channel transcript expressed in rat testis encodes a delayed rectifier K⁺ channel.



Figure 8. Results from 5' rapid amplification of cDNA ends. Typical results are shown. The sizes of polymerase chain reaction (PCR)-amplified products were calculated based on their mobility in a 1.2% agarose gel containing ethidium bromide relative to the DNA fragments of the 123 bp marker ladder (M). Rat testis cDNA was amplified using 5' adaptor primer (AP1) and 3' gene-specific primer (RVGKCE1) (lane 1). A 1 μ l aliquot of the PCR reaction from lane 1 was reamplified with 5' nested adaptor primer (AP2) and 3' gene-specific primers RVGKCE3 (lane 2) and RVGKCE4 (lane 3).

5' RACE

The failure to obtain a PCR product with extreme 5' primer pair RAKC6F/RAKC6R suggests that the 5' end of the K^+ channel transcript in rat testis is structurally different from Kv1.3. Therefore, the method for rapid amplification of cDNA ends (RACE) was employed in an attempt to amplify the 5' sequences of the rat testis cDNA. The gene-specific primers used in these reactions were RVGKCE1 or RVGKCE2. A smear of EB staining was observed when the products of these 5' RACE reactions were analysed by electrophoresis through an agarose gel (e.g. Figure 8, lane 1). According to the supplier of the reagents used for RACE, a smear is most often seen when amplifying cDNA from a multigene family in which the gene-specific primer may simultaneously amplify several highly homologous cDNA.

To obtain a single product, an aliquot of this RACE reaction was used in a second PCR using a nested 5' primer (AP2) and two different nested 3' gene-specific primers (RVGKCE3 and RVGKCE4) (Figure 8, lanes 2 and 3). The PCR reactions generated single robust products of 300 or 400 bp with the 3' gene specific primers. Both products were purified and subjected to direct DNA sequencing. A unique sequence could not be obtained with either product. The inability to obtain a single sequence was not due to lower amounts of PCR products amplified from the RACE reaction (see Figure 8, lanes 2 and 3). Rather, the chromatogram from the automated DNA sequencer suggested that multiple cDNA sequences were imposed on each other.

These data suggest that multiple related K^+ channel sequences were present in rat testis.

Discussion

Our interest in mammalian sperm K^+ channels stems from four sets of observations. First, it was reported that exposure of human spermatozoa to progesterone results in the activation of a monovalent cation channel (Foresta *et al.*, 1993). Second, it was found that progesterone-regulated ion movements in human spermatozoa are involved in cytosolic alkalinization (Garcia and Meizel, 1996). Third, altered sperm membrane potential in many infertile men is associated with defective K^+ transport (Calzada and Tellez, 1997). Fourth, we observed that: (i) progesterone binding to the human sperm surface non-nuclear progesterone receptor activates K^+ channels; (ii) K^+ channel inhibitors block the acrosome reaction induced by model zona ligands containing mannose; and (iii) inhibition of progesterone-stimulated acrosome loss is a biomarker for the effects on sperm function of environmental exposure to heavy metals such as lead (Benoff, 1999; Benoff *et al.*, 1999; A.Jacob, I.R.Hurley, G.M.Centola, G.W.Cooper and S.Benoff, unpublished observations).

Molecular characterization of K^+ channels in spermatozoa or testis has been limited. To our knowledge only four such channels have been reported. First, sea urchin spermatozoa express K^+ channels on their head (Guerrero *et al.*, 1987) and flagellar (Lievano *et al.*, 1985; Labarca *et al.*, 1996) plasma membranes. Their electrophysiological characterization indicated that sea urchin sperm K^+ channels exhibit a relatively large unitary conductance, suggesting that they are related to voltage-gated Ca^{2+} -activated (Latorre and Miller, 1983) or delayed rectifier (Conti and Neher, 1980) K^+ channels (for discussion, see Lievano *et al.*, 1985). Second, a hyperpolarization-activated K^+ channel (SPIH) regulated by voltage and adenosine 3',5'-cyclic monophosphate was recently cloned in sea urchin spermatozoa (Gauss *et al.*, 1998). This channel activity is confined to the sperm flagellum, suggesting that SPIH channels are involved in flagellar beating. Third, a cyclic nucleotide-gated (CNG) channel has been cloned from bovine testis (Weyand *et al.*, 1994). CNG K^+ channels have also been shown in sea urchin spermatozoa (Cook and Babcock, 1993; Labarca *et al.*, 1996). Fourth, a pH-sensitive K^+ channel (Slo3) has been identified in mammalian spermatocytes (Schreiber *et al.*, 1998) that is highly homologous to the large conductance Ca^{2+} -activated (BK) K^+ channel, Slo1. BK K^+ channels are expressed in spermatogenic cells from rat testis (Chan *et al.*, 1998). Thus, this report is the first to document the expression of delayed rectifier K^+ channels in mammalian testis and spermatozoa.

Multiple mammalian K^+ channel gene families have been identified using a *Drosophila Shaker* K^+ channel cDNA probe (Baumann *et al.*, 1988; Stuhmer *et al.*, 1989). The Kv1 family is the best characterized and its members primarily encode delayed rectifiers when expressed *in vitro* (Pongs, 1992; Salkoff *et al.*, 1992). The Kv1 gene family is comprised of at least eight members, Kv1.1–Kv1.8 (Lock *et al.*, 1994). The rat testis K^+ channel sequence described herein showed 96–98%

nucleotide sequence identity to the corresponding regions of Kv1.3 cDNA and a 99% amino acid sequence identity to Kv1.3 protein.

Our in-situ RT-PCR localization of K⁺ channel transcripts indicated that the channel mRNA is observed in the cytoplasm of primary spermatocytes in stages VII through IX/X of spermatogenesis, when actin interacts with the developing acrosome granule to regulate shaping of the acrosome around the sperm nucleus and establish the mature acrosome and head shape (Leblond and Clermont, 1952; Vogl, 1989). These stage- and cell type-specific findings on K⁺ channel gene expression are in contrast to similar studies performed to detect the mRNA encoding a component of another ion channel participating in the human sperm acrosome reaction, the α -1C subunit of L-type voltage-dependent Ca²⁺ channels (Goodwin *et al.*, 1998, 2000). L-type α -1C transcripts were amplified in all cell types found within the rat seminiferous tubules but not in the interstitial spaces. Further, accumulation of L-type α -1C transcripts was not limited to a particular stage of the cycle of the rat seminiferous epithelium.

Nevertheless, as observed for Ca²⁺ channel transcripts (Goodwin *et al.*, 1998, 2000), K⁺ channel transcripts are also present in post-meiotic elongating spermatids and mature spermatozoa. Hybridization signal was not detected in intervening stages (e.g. round spermatids) of sperm development. This observation is somewhat perplexing. Since the S4F/S6R primer pair used to amplify K⁺ channel in rat testis is derived from a highly conserved region of different classes of K⁺ channels, one possible explanation for this discrepancy is that the transcript observed in the cytoplasm of primary spermatocytes is distinct from that present in the elongating spermatids. In this regard, we note that a unique form of actin mRNA is expressed solely in the post-meiotic stages (Gu *et al.*, 1996).

Our Northern blot analysis provides evidence for expression of at least three Kv1-related transcripts in rat testis, i.e. 5, 4.2 and 2.4 kb. Multiple related mRNA for K⁺ channel proteins have also been observed in somatic tissues in the rat (Baumann *et al.*, 1988). These differentially expressed transcripts are either product of alternate splicing of the primary transcript or transcripts from independent genes. Kv channels in mammalian systems arise primarily from independent genes, and in most cases a single exon specifies the entire K⁺ channel coding region (Salkoff *et al.*, 1992; Christie, 1995). However, diversity of *Drosophila Shaker* channels is generated through alternate splicing to produce different mRNA from a single large transcription unit (Kamb *et al.*, 1987; Pongs *et al.*, 1988; Schwarz *et al.*, 1988). Although less prominent, K⁺ channel genes are alternatively spliced to produce different subunits [e.g. Kv4.3 (Ohya *et al.*, 1997), Kv3.1 (Perney *et al.*, 1992)]. At present, it is not clear whether the differentially expressed transcripts are products of multiple independent genes or of alternate splicing.

Mammalian somatic Kv channels, such as Kv1.3, are modulated by phosphorylation, i.e. K⁺ currents are upregulated by PKC (Cai *et al.*, 1992; Payet and Dupuis, 1992; Chung and Schlichter, 1997) and down-regulated by TK activity (Holmes *et al.*, 1996; Bowlby *et al.*, 1997). The Kv1.3 protein expressed in rat testis, based on the deduced amino acid sequence,

potentially can be modulated in a similar manner. It is therefore important to note that, in human sperm, the K⁺ channel proteins co-localize with surface plasma membrane non-nuclear progesterone receptors (Benoff *et al.*, 1995, 1999; Jacob *et al.*, 1998a). Progesterone-stimulated Ca²⁺ influx is mediated by PKC (Foresta *et al.*, 1995) and TK activity prevents voltage-induced current facilitation (Arnoult *et al.*, 1997). These findings, together with the fact that inhibitors of delayed rectifier K⁺ channels block progesterone-stimulated acrosome loss, suggest a role for these channels in the mammalian acrosome reaction (Benoff, 1999).

Preliminary findings, based on inter-male variations in inhibitor sensitivities of progesterone-stimulated acrosome loss, suggest that multiple isoforms of the human homologue of rat Kv1.3 exist and that each man expresses only one isoform in his ejaculated spermatozoa (Jacob *et al.*, 1998b; Benoff, 1999; S.Benoff and G.M.Centola, unpublished data). We believe that such diversity is of clinical importance. We postulate that differential expression of these K⁺ channel isoforms could underlie inter-male differences in acrosome response to progesterone exposure as well as susceptibility to environmental lead exposures. Experiments are in progress to test this hypothesis.

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Male infertility and environmental exposure to lead and cadmium

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Humans are exposed occupationally and environmentally to metal aerosols including lead (Pb²⁺) and cadmium (Cd²⁺). These toxicants accumulate in male reproductive organs. Epidemiological studies have been equivocal about effects of Pb²⁺ and Cd²⁺ on hormone concentrations, male fertility and sperm parameters. Comparison of Pb²⁺ and Cd²⁺ concentrations in fertile and infertile men are problematic. Problem areas include failure to control confounding variables, but genetic polymorphisms as in somatic diseases may modulate Pb²⁺ and Cd²⁺ damage. Multiple calcium (Ca²⁺) and potassium (K⁺) channel isoforms have been identified in human testes and spermatozoa. These Ca²⁺ and K⁺ channels are involved in early events of acrosome reactions. Ca²⁺ channel are susceptible to Cd²⁺ poisoning and K⁺ channels to Pb²⁺. These channels offer entry paths for metallic toxicants into mature spermatozoa. Ion channel polymorphisms may cause differential sensitivities to Cd²⁺ and Pb²⁺, explaining in part prospective blinded studies showing high Cd²⁺ in varicocele-related human infertility and high Pb²⁺ in unexplained infertility. In both forms of male infertility the ability to undergo an acrosome reaction decreases. Reverse transcriptase–polymerase chain reaction assays for Ca²⁺ and K⁺ channel isoforms may identify susceptibility subgroups with lower resistance to environmental exposures.

Key words: acrosome reaction/cadmium/ion channels/lead/male infertility

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Introduction

In recent years, there has been an increasing interest in the contribution of occupational and environmental exposures to toxic pollutants to declining sperm concentrations and human male infertility (Carlsen *et al.*, 1992; Auger *et al.*, 1995; Adamopoulos

et al., 1996; Becker and Berhane, 1997). We recognize that at least one of these studies (Carlsen *et al.*, 1992) has been criticized because it fails to consider that sperm counts clearly differ by geographical location (Fisch and Goluboff, 1996; Paulsen *et al.*, 1996). Nonetheless, geographic variations in semen quality (Fisch and Goluboff, 1996; Becker and Behane, 1997) may still be influenced by environmental factors (Fisch and Goluboff, 1996) which exhibit considerable variation between climatic seasons (Sram *et al.*, 1996). More importantly, environmental factors differ between areas (Friberg and Vahter, 1983; Svensson *et al.*, 1987; Buchancova *et al.*, 1994; Sram *et al.*, 1996), with higher amounts of pollutants closer to sources of industrialization (Benin *et al.*, 1999).

Unfortunately, relatively few studies have systematically addressed the impact of environmental exposures on human health in general (Buchancova *et al.*, 1994; Staessen *et al.*, 1996) or on human reproductive function (El-Zohairy *et al.*, 1996; Sram *et al.*, 1996). What limited data that does exist suggests that exposure to high values of airborne fine particles is associated with decreased semen quality, i.e. increased aberrant morphology and motility. Heavy and transition metal ions, e.g. cadmium (Cd²⁺), cobalt (Co²⁺), copper (Cu²⁺), iron (Fe^{2+/3+}), lead (Pb²⁺), mercury (Hg²⁺), magnesium (Mg²⁺), manganese (Mn²⁺), nickel (Ni²⁺)

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and selenium ($\text{Se}^{2+}/4+$), are common components of such airborne particulates that are within the respirable range (Ragan and Mast, 1990). Pb^{2+} and Cd^{2+} preferentially accumulate in male reproductive organs (Danielsson *et al.*, 1984; Oldereid *et al.*, 1993; Jackson *et al.*, 1995). An increase in Pb^{2+} and Cd^{2+} often occur simultaneously (Stachel *et al.*, 1989). This review focuses on the effects of Pb^{2+} and Cd^{2+} . Our studies, which are summarized herein, indicate that Pb^{2+} and Cd^{2+} exposures are of particular importance to the production of the infertile state.

Effect of occupational exposure to metal ions

The correlation of trace metal exposures with human sperm production and function has been studied mainly in men occupationally exposed to high concentrations of airborne metal particulates (Danielsson *et al.*, 1984; Assennato *et al.*, 1986; Stachel *et al.*, 1989; Gennart *et al.*, 1992b; Bonde, 1993). The earliest studies were triggered by reports that semen values of another class of environmental toxicants, halogen-containing hydrocarbon pesticides, were associated with decreases in sperm counts and motility, increased percentages of spermatozoa with morphological abnormalities and male infertility (Wong *et al.*, 1979; Dougherty *et al.*, 1986; Ratcliffe *et al.*, 1987; Wagner *et al.*, 1990).

The possible xenobiotic exposures associated with industrial metal aerosols include metals such as aluminum (Al^{3+}), Cd^{2+} , $\text{Fe}^{2+}/^{3+}$, chromium (Cr^{2+}), Ni^{2+} , and Pb^{2+} , as well as nitrous gases and ozone (Stern *et al.*, 1986; Mortensen, 1988). Metal workers (e.g. welders, smelters, men engaged in the manufacture of alkaline batteries) comprise 0.5–2.0% of the general working population (Stern *et al.*, 1986). There is epidemiological evidence that exposure to industrial metal aerosols may be detrimental to the male reproductive system; fertility of men employed in the metal industry is apparently reduced when compared with men in other types of work. These statements are based upon studies reporting decreased family size, delay in time to conception and reduced sperm quality in metal workers (Bonde, 1993; Spinelli *et al.*, 1997). These problems have been correlated with both exposure dose and length of exposure (Bonde, 1990a), and are not reversible by short periods of non-exposure (Bonde, 1990b). However, despite these findings, attempts to correlate occupational exposure to Pb^{2+} and Cd^{2+} with semen quality and/or infertility have produced conflicting results.

Blood plasma and seminal plasma Pb^{2+} concentrations are elevated in exposed workers (Stachel *et al.*, 1989; Telisman *et al.*, 1990; Ng *et al.*, 1991; Gennart *et al.*, 1992a; Keck *et al.*, 1995; Aribarg and Sukcharoen, 1996; Robins *et al.*, 1997; see Table I). A positive relationship was detected between blood and seminal plasma Pb^{2+} concentrations (Aribarg and Sukcharoen, 1996). In some studies, evidence is presented suggesting that men with a long duration of Pb^{2+} exposure in the workplace may have reduced fertility (Lancranjan *et al.*, 1975; Gennart *et al.*, 1992b; Lin *et al.*, 1996). However, in other studies, Pb^{2+} exposure has not been reported to decrease fertility (Coste *et al.*, 1991) or only exhibits a weak association with infertility (Robins *et al.*, 1997). Dose-response relationships and/or causal mechanisms remain to be defined (Winder, 1993). Seminal plasma Pb^{2+} concentrations and fertility status have not been correlated (Keck *et al.*, 1995).

Endocrine dysfunction has (Ferioli *et al.*, 1989; MacGregor and Mason, 1990; Ng *et al.*, 1991) and has not (Lancranjan *et al.*, 1975; Assennato *et al.*, 1986) been observed in men occupationally exposed to Pb^{2+} . Where occurring, Pb^{2+} -associated changes in hormone concentrations were thought to be mediated at the hypothalamic-pituitary level (Gustafson *et al.*, 1989; Ng *et al.*, 1991). These data are consistent with findings in animal models, where the primary toxic action of Pb^{2+} on male reproduction appears to be disruption of the hypothalamus-pituitary-testicular axis (Sokol *et al.*, 1985, 1994; Sokol, 1987; Kempinas *et al.*, 1994).

The majority of reports indicate that workers exposed to Pb^{2+} exhibit decreased sperm density and a high rate of teratozoospermia (Lancranjan *et al.*, 1975; Hu *et al.*, 1992; Lerda, 1992; Xuezhi *et al.*, 1992; Robins *et al.*, 1997) and may also present with a decreased sperm count (Assennato *et al.*, 1986). An inverse correlation between blood Pb^{2+} concentrations and decreased semen quality has been observed (Lancranjan *et al.*, 1975; Telisman *et al.*, 1990). The study by Telisman *et al.* (1990) also suggested that reproductive toxicity may occur at blood Pb^{2+} concentrations below the permissible exposure limit of 40 $\mu\text{g}/\text{dl}$. Thus, current occupational standards may not be sufficiently stringent (Winder, 1993).

In some studies, however, no relationship was detected between seminal plasma Pb^{2+} concentrations and semen profiles (Aribarg and Sukcharoen, 1996; Hovatta *et al.*, 1998). There are three possible explanations for this failure. Firstly, exposure levels were apparently very low as blood Pb^{2+} concentrations were generally <40 $\mu\text{g}/\text{dl}$, the recommended limit of exposure for workers in an occupational setting as defined by the World Health Organization (WHO, 1980). Secondly, in some cases the men studied had only short-term exposure to Pb^{2+} (Aribarg and Sukcharoen, 1996). As in animal models (Kempinas *et al.*, 1994), duration of Pb^{2+} exposure appears to be an important factor when searching for a correlation between Pb^{2+} and changes in semen parameters (Lerda, 1992). Thirdly, measurements of metal ion concentrations in seminal plasma may be of less significance in the examination of environmental effects than direct measurements of metal ion concentrations in spermatozoa themselves. As an example of the latter, Hovatta *et al.* (1998) reported that sperm motility was inversely correlated with aluminium in spermatozoa but not in seminal plasma.

Whether Cd^{2+} concentrations in blood and seminal plasma are elevated following occupational exposures is still not clear (Table I). Early studies indicated that, based on autopsy findings, spermatogenesis was reduced in workers exposed to Cd^{2+} (Smith *et al.*, 1960) and that the family size was smaller than average (Favino *et al.*, 1968). More recent studies did not support these findings. Cd^{2+} exposures have not been associated with a significant reduction in semen quality (Tielmans *et al.*, 1999) or with a decreased probability of a live birth (Gennart *et al.*, 1992b). In contrast, occupational exposure to Cd^{2+} has been reported to increase the risk for prostate cancer (Elghany *et al.*, 1990). This is consistent with observations indicating that Cd^{2+} concentrations are increased in prostate neoplasms, compared with normal tissue (Ogunlewe and Osegbe, 1989; Brys *et al.*, 1997) and studies *in vitro* and in animal models have demonstrated a relationship

Table I. Effects of occupational exposures on the levels of Pb2+ and Cd2+ in blood and seminal plasma

	Unexposed	Exposed	Source
Pb2+ (blood)	13.1 ± 3.5 µg/dl		Plechaty <i>et al.</i> (1977)
	23 ± 14 µg/dl	29–100.5 µg/dl*	Lancrajan <i>et al.</i> (1975)
	20.8 ± 2.8 µg/dl	50.5 ± 9.6 µg/dl*	Feroli <i>et al.</i> (1989)
	6.7 – 20.8 µg/dl	11.7 – 104.0 µg/dl*	Telisman <i>et al.</i> (1990)
	8.3 ± 2.8 µg/dl	35.2 ± 13.2 µg/dl*	Ng <i>et al.</i> (1991)
	20.9 ± 11.1 µg/dl	51.0 ± 8.0 µg/dl*	Gennart <i>et al.</i> (1992a)
	10.4 ± 3.3 µg/dl	46.3 ± 11.1 µg/dl*	Gennart <i>et al.</i> (1992b)
	23.5 ± 1.4 µg/100 g	86.6 ± 0.6 µg/100 g*	Lerda (1992)
	23.6 ± 47.1 µg/dl	47.1 ± 31.0 µg/dl*	Xuezhi <i>et al.</i> (1992)
	16.9 ± 2.6 µg/dl	26.7 ± 2.1 µg/dl*	El-Zohairy <i>et al.</i> (1996)
Pb2+ (semen)		(Fertile)	
		37.0 ± 4.9 µg/dl*	
		(Infertile)	
	27.9 ± 2.5 µg/dl	45.0 ± 9.4 µg/dl*	Aribarg and Sukcharoen (1996)
	5.9 ± 2.7 µg/dl		Plechaty <i>et al.</i> (1977)
	16.5 ± 17.7 µg/dl	66.5 ± 83.6 µg/dl*	Xuezhi <i>et al.</i> (1992)
	11.0 ± 1.9 µg/dl	21.9 ± 2.0 µg/dl*	El-Zohairy <i>et al.</i> (1996)
		(Fertile)	
		25.8 ± 3.2 µg/dl*	
		(Infertile)	
Cd2+ (blood)	18.6 ± 3.3 µg/dl	38.3 ± 10.5 µg/dl*	Aribarg and Sukcharoen (1996)
	0.22 ± 0.21 µg/dl	0.16 ± 0.14 µg/dl	Xuezhi <i>et al.</i> (1992)
Cd2+ (semen)	0.04 ± 0.03 µg/dl	0.05 ± 0.05 µg/dl	Xuezhi <i>et al.</i> (1992)
	0.38 ± 0.64 µg/l	3.28 ± 0.40 µg/l*	Keck <i>et al.</i> (1995)

*Statistically significant increase, $P < 0.01$ – 0.0001 .

between Cd2+ and malignant transformation of prostate epithelial cells (Voeller *et al.*, 1991; Waalkes and Rehms, 1994).

The controversy concerning whether or not the fertility potential of Pb2+ and/or Cd2+-exposed men is decreased may not be resolved in the near future as the developmental risks, e.g. abortions, stillbirths, postnatal deaths, congenital malformations (Sallmen *et al.*, 1992; Xuezhi *et al.*, 1992), from occupational Pb2+ exposures appears to be of greater concern to employers than male reproductive effects (Paul and Kurtz, 1994). This appear also true of investigators studying the effects of environmental exposures (Sram *et al.*, 1996).

Sources of Pb2+ and Cd2+ found in the environment

Environmental discharges of Pb2+ are a major concern as Pb2+ wastes are tracked as hazardous materials. Pb2+ is probably the most common neurotoxin and suspected reproductive toxicant present in the environment at high concentrations as the result of the use of petroleum products (Hutton and Symon, 1986) and

demand for lead-acid batteries, the latter requiring mining, smelting and recycling of Pb2+ (Lave *et al.*, 1995). Combustion of fossil fuels (petroleum, coal) and municipal refuse also contribute to airborne Cd2+ pollution (Hutton and Symon, 1986; Ragan and Mast, 1990). As for Pb2+, regional concentrations of Cd2+ may be higher near industrial operations, such as smelters and battery and paint factories (Benin *et al.*, 1999). In addition, the public may be unwittingly exposed to Pb2+ or Cd2+ via contaminated food or paper (Wu *et al.*, 1995) and cosmetics and herbal folk remedies (Wong and Koh, 1986; Lockitch, 1993). The major risk factors for Pb2+ toxicity include age, nutrition (deficiencies in Ca2+, Fe2+/3+ and Zn2+), housing and socioeconomic status and, importantly, smoking (Goyer, 1993).

Cigarette smoking is an important confounding variable when considering the effects of both environmental Pb2+ and Cd2+ exposures on human health (Sram *et al.*, 1996). Cigarette smoke is a major source of airborne environmental Pb2+ and Cd2+ exposures. A single cigarette contains 0.6–2.0 µg Pb2+ (Chiba and Masironi, 1991) and 1–4.5 µg Cd2+ (Chia *et al.*, 1994b;

Saldivar *et al.*, 1991) and at least one tenth of the metal content of a cigarette is inhaled (Elinder *et al.*, 1985).

Examination of the effects of environmental exposure to metal ions

Agents which are reproductive toxicants in the workplace are found in human semen, e.g. Cd²⁺, dibromochloropropane, lithium, and pesticides (Gagnon, 1988). However, the role of increased seminal plasma trace metal concentrations in regulating reproductive function in the occupationally unexposed male is poorly understood. Geographic differences in the amount of naturally occurring Cd²⁺ have been correlated with incidence rates of prostate cancer (Garcia Sanchez *et al.*, 1992; Angwafo, 1998). In contrast, it has been difficult to identify an association between environmental metal exposures and male infertility. Since the desire for reproduction occurs only intermittently, environmental exposures that interfere with reproduction may go unnoticed (Mattison *et al.*, 1990).

Tobacco smoke is the best studied environmental exposure. Cigarette smoking impairs both female and male fecundity (Zenses, 1995; Hughes and Brennan, 1996; Curtis *et al.*, 1997). The decrease in fecundity in men who smoke cigarettes may be the result of reductions in semen volume, sperm density, sperm motility, normal sperm morphology and/or sperm viability (Saaranen *et al.*, 1987b; Radcliffe *et al.*, 1992; Chia *et al.*, 1994a,b; Vine *et al.*, 1994; Omu *et al.*, 1995; Sofikitis *et al.*, 1995; Vine, 1996; Merino *et al.*, 1998; Rubes *et al.*, 1998). An increase in sperm aneuploidy, e.g. Y disomy, has recently been noted (Rubes *et al.*, 1998). The latter is consistent with an increase in proportion of female offspring in men exposed to Pb²⁺ (Dickinson and Parker, 1994). The outcome of sperm function assays was significantly lower in men who smoke, compared with non-smoking controls (Sofikitis *et al.*, 1995). Cigarette smoke may also potentiate the effects of other suspected reproductive toxicants, e.g. caffeine (Bolumar *et al.*, 1997).

Although blood concentrations of Cd²⁺ and Pb²⁺ may be increased in smokers (Brockhaus *et al.*, 1983; Friberg and Vahter,

Table II. Comparison of Pb²⁺ and Cd²⁺ concentrations in blood and seminal plasma in men who smoke cigarettes and non-smoking control subjects

	Non-smokers	Smokers	Source
Pb ²⁺ (blood)	92.0 µg/l	99.2 µg/l	Brockhaus <i>et al.</i> (1983)
		7.09 µg/l	Chia <i>et al.</i> (1994b)
Pb ²⁺ (semen)		12.9 µg/l	Chia <i>et al.</i> (1994b)
	0.24–0.38 µg/l	0.24–0.34 µg/l	Oldereid <i>et al.</i> (1994)
		(<1 pack/day)	
		0.22–0.39 µg/l	
		(>1 pack/day)	
Cd ²⁺ (blood)	0.44 µg/l	1.85 µg/l*	Brockhaus <i>et al.</i> (1983)
	0.63 ± 0.44 µg/l	1.66 ± 0.93 µg/l	Moreau <i>et al.</i> (1983)
	0.24 ± 0.09 µg/l	0.31 ± 0.06 µg/l*	Saaranen <i>et al.</i> (1989)
		(<1 pack/day)	
		0.39 ± 0.05 µg/l*	
		(>1 pack/day)	
	0.84 µg/l	1.55 µg/l*	Chia <i>et al.</i> (1994b)
Cd ²⁺ (semen)	0.19 ± 0.21 µg/l	0.23 ± 0.17 µg/l	Saaranen <i>et al.</i> (1989)
		(<1 pack/day)	
		0.40 ± 0.40 µg/l	
		(>1 pack/day)	
	0.54 µg/l	0.86 µg/l	Chia <i>et al.</i> (1994b)
	0.14–0.32 µg/l	0.19–0.38 µg/l	Oldereid <i>et al.</i> (1994)
		(<1 pack/day)	
		0.29–0.49 µg/l*	
		(>1 pack/day)	

*Statistically significant increase, $P < 0.05$ – 0.001 .

Table III. Concentrations of Pb2+ and Cd2+ reported in blood and semen from fertile and infertile men

	Fertile	Infertile	Source
Pb2+ (blood)		6.5 ± 5.4 µg/l	Chia <i>et al.</i> (1992)
	16.9 ± 2.6 µg/dl	29.3 ± 3.8 µg/dl*	El-Zohairy <i>et al.</i> (1996)
Pb2+ (semen)	7.4 ± 0.7 µg/l		Pleban and Mei (1983)
	255 ± 123 µg/l	243 ± 179 µg/l	Umeyama <i>et al.</i> (1986)
	1.7 ± 1.0 µg/l	3.6 ± 3.2 µg/l*	Saaranen <i>et al.</i> (1987a)
	8 ± 1.1 µg/l	1.2 ± 1.2 µg/l	Abou-Shakra <i>et al.</i> (1989)
		(Oligospermic)	
		6 ± 4 µg/l	
		(Severely oligospermic)	
		1.0 ± 1.3 µg/l	
		(Azoospermic)	
	5.61 ± 0.53 µg/l	11.18 ± 0.62 µg/l*	Jockenhovel <i>et al.</i> (1990)
	7.7 ± 5.6 µg/l		Noack-Fuller <i>et al.</i> (1993)
	11.0 ± 1.9 µg/dl	19.6 ± 2.4 µg/dl*	El-Zohairy <i>et al.</i> (1996)
Cd2+ (blood)		1.35 ± 1.0 µg/l	Chia <i>et al.</i> (1992)
Cd2+ (semen)	0.48 ± 0.04 µg/l		Pleban and Mei (1983)
	5 ± 6 µg/l	13 ± 12 µg/l*	Umeyama <i>et al.</i> (1986)
	0.34 ± 0.16 µg/l		Noack-Fuller <i>et al.</i> (1993)
	0.38 ± 0.64 µg/l	0.43 ± 0.69 µg/l	Keck <i>et al.</i> (1995)

*Statistically significant increase, $P < 0.05$ – 0.005 .

1983; Moreau *et al.*, 1983; Xu *et al.*, 1993; see Table II), it is more likely that the negative effects of smoking on semen quality are a result of the observed trend for Cd2+ in tobacco smoke to accumulate in the genital tract of smokers (Saaranen *et al.*, 1989; Pacifici *et al.*, 1993; Oldereid *et al.*, 1994; Keck *et al.*, 1995; Omu *et al.*, 1995; see Table II). In contrast, seminal plasma Pb2+ concentrations appear unaffected by smoking status (see Table II).

Not only are smokers at risk. A positive correlation has been observed between the degree of passive exposure to cigarette smoke and the concentration of components of cigarette smoke in seminal plasma (Pacifici *et al.*, 1995).

Subfertile or infertile men may be more sensitive to the deleterious effects of cigarette smoke than fertile men (Zenses, 1995). Risk factors for unexplained dyspermia in infertile men include smoking (Parazzini *et al.*, 1993). Total sperm count and sperm motility are lower in oligozoospermic men who smoke than in oligozoospermic non-smokers (Rantala and Koskimies, 1987; Vine *et al.*, 1996). An acrosome reaction insufficiency was first suggested from the significant decrease in penetration of zona-free hamster oocytes by spermatozoa from infertile men who smoked cigarettes, compared with infertile men who were non-smokers (Close *et al.*, 1990). A reduced inducibility of the acrosome reaction in subfertile men who smoke has since been confirmed (El Mulla *et al.*, 1995). The latter is consistent with reports of increased 'round-headed' spermatozoa, which lack an acrosome

(Zamboni, 1987; Escalier, 1990), in the semen of smokers (Rubes *et al.*, 1998). Hormonal alterations may occur (Gerhard *et al.*, 1992). Anti-sperm antibody production is increased in smokers (Omu *et al.*, 1997). An improvement in semen quality has been noted when subfertile men quit smoking (Vine, 1996). The concept of infertility patients as forming 'susceptibility subgroups' is discussed in greater detail later in this review (see section on genetic determinants of susceptibility below).

Metal ion concentrations in fertile and infertile men

It has long been suggested that at least one half of the cases of human male infertility of unknown aetiology may be attributable to various environmental and occupational exposures (Steen and Pangkahila, 1984a,b; Gagnon, 1988). Therefore, infertility patients have often been used in attempts to investigate the role of environmental metals. Data from such studies may be skewed as a result of selection bias. Not all couples experiencing infertility seek medical intervention (Olsen *et al.*, 1998).

Semen from men presenting for fertility evaluations generally contain only low concentrations of Pb2+ and Cd2+ (Noack-Fuller *et al.*, 1993). Nevertheless, a considerable body of evidence indicates that decreased concentrations of Zn2+ in seminal plasma and semen occur in some forms of infertility (Marmar *et al.*, 1975; Umeyama *et al.*, 1986; Saaranen *et al.*, 1987a; Hurley *et al.*, 1997;

S.Benoff, A.Hershlag and I.R.Hurley, unpublished observations). In contrast, the majority of reports suggest that environmental exposures to Pb²⁺ do not significantly contribute to male infertility. In at least half the published studies, seminal plasma Pb²⁺ values have been reported to be similar in fertile and infertile men (Umeyama *et al.*, 1986; Abou-Shakra *et al.*, 1989; see Table III).

Many studies have failed to find any significant differences in the seminal plasma Cd²⁺ concentrations of fertile and infertile men (Keck *et al.*, 1995). In contrast, in other studies, seminal plasma Cd²⁺ concentrations were found to be increased in infertile, compared with fertile, men (Umeyama *et al.*, 1986; Saaranen *et al.*, 1987a; Jockenhovel *et al.*, 1990). In addition, increases in blood plasma Cd²⁺ concentrations in infertile men have been associated with teratozoospermia (Chia *et al.*, 1992) and an inverse correlation between blood plasma Cd²⁺/seminal plasma Cd²⁺ and sperm density and semen volume has been reported (Xu *et al.*, 1993). These conflicting findings are summarized in Table III.

Confounding variables

Confounding variables are likely to be responsible for many of the apparent contradictions between similar studies cited in the preceding section. Groups of environmental toxicologists have been convinced of this, and have proposed guidelines for controlling such variables (Tas *et al.*, 1996; Wyrobek *et al.*, 1997). Central to these strategies is use of multiple study endpoints (Perreault *et al.*, 1989), including biomarkers (Smith and Suk, 1994), to assess exposures to other toxicants which may produce or mask conditions induced by the chemical(s) under study. The extent of knowledge of a particular chemical's toxicology and its underlying molecular biology insofar as it relates to the outcome measure under study will determine the comprehensiveness of the list of confounding variables and endpoints.

Depending upon outcome measure, endpoints may include questionnaires to gather medical and reproductive histories, information on lifestyle factors, e.g. smoking, herbal therapy (Ondrizek *et al.*, 1999) and exposures to unrelated toxicants. Physical examinations of subjects are useful in revealing physical conditions affecting male fertility which may not be evident from questionnaires, including varicocele and small testicular size. A standardized classical semen analysis, computerized sperm morphometry and motility together with blood hormone concentrations are recommended both to characterize the effects of a particular exposure and to identify sub-populations, which presumably differ with respect to some confounding variable. Choice of control groups are also a major issue, e.g. see Favino *et al.* (1968) who used Pb²⁺-exposed men as controls for the effects of Cd²⁺ on reproductive hormones.

The state of knowledge of heavy metal confounders is relatively satisfactory. Concentrations of various metal ions have been assayed in the same tissues in animal models. This has revealed that transport and equilibrium concentrations of heavy metals can be altered by concentrations of other metals administered in the course of both acute and chronic exposure studies. For example, similarities of the ionic size and normal ionic charge between Cd²⁺, Ca²⁺ and Zn²⁺ with Pb²⁺ mean that both Cd²⁺ and Pb²⁺

can pass the gut/blood barrier by binding to Ca²⁺ and Zn²⁺ transport sites on the surface of intestinal villae (Goyer, 1997). High amounts of Ca²⁺ in diet are protective against both Cd²⁺ and Pb²⁺ poisoning. However, gaps remain. Measurements of effects of Pb²⁺ upon sperm fertility outcome measures, such as progesterone-induced acrosome loss (see section on ion channels and response to environmental metal exposures below), therefore require assessment of the inter-reacting metal confounders, Cd²⁺, Zn²⁺ and Ca²⁺ in the same samples. These measurements must be both accurate and sensitive, as different methods appear to produce discordant results (e.g. colorimetric versus graphite furnace atomic absorption spectroscopy; Fuentes *et al.*, 1981 and Jeyendran *et al.*, 1989 versus Canale *et al.*, 1986, Xu *et al.*, 1993 and Hurley *et al.*, 1997). Other confounding variables which affect the availability of metals may be addressed using conventional assays. These include transport and storage proteins, and components of diet. For example, high phosphate in the diet potentiates Pb²⁺ poisoning, by forming insoluble complexes with dietary Ca²⁺.

There is also a need for reliable molecular biomarkers to assess individual susceptibilities to heavy metal toxicity (Todd *et al.*, 1996). Individual variability could explain why, though blood Pb²⁺ concentrations can clearly be increased by environmental exposures (Sram *et al.*, 1996), population studies of effects of chronic Pb²⁺ exposure upon fertility-related outcome measures have not produced consensus. The goal therefore is to develop a group of markers similar to the genetically determined polymorphisms in enzymes susceptible to Pb²⁺ inhibition, which have been suggested to be useful for predicting renal toxicity (Doss *et al.*, 1982; Todd *et al.*, 1996). We suggest, however, that the most important confounding variable is the choice of the population(s) to be studied.

Genetic determinants of susceptibility

There is an increasing awareness in environmental toxicology for factors that predispose individuals to be sensitive to toxicant exposure (Anderson *et al.*, 1994). One of the primary factors leading to the existence of this susceptibility is genetic polymorphisms.

Somatic diseases

Susceptibility subgroups associated with genetic polymorphisms have been identified in a variety of somatic cell diseases, including sickle cell anaemia (Lu and Steinberg, 1996), tobacco-related lung cancer and gastric cancer (Hirvonen, 1995), breast or ovarian cancer (Suk *et al.*, 1996) and prostate cancer (Kantoff *et al.*, 1998). These polymorphisms may take the form of unstable simple DNA repeats (Panzer *et al.*, 1995) or functional enzyme polymorphisms with marked inter-individual variation (Caraco, 1998) that mediate oxidative metabolism and detoxification reactions, e.g. cytochrome P-450 (CYP), monamine oxidase, superoxide dismutase and glutathione S-transferase (GST) (Hirvonen, 1995; Raunio *et al.*, 1995; Watanabe, 1998).

Infertility

A large portion of male infertility is thought to have an underlying genetic basis, e.g. gonadotrophin-releasing hormone (GnRH) deficiency, spermatogenic failure and both obstructive (e.g.

congenital absence of the vas deferens) and non-obstructive azoospermia (e.g. associated with Y deletions) (Mak and Jarvi, 1996). An association between expression of certain human leukocyte antigen antigens (e.g. HLA-A28 and Bw40) and azoospermia has also been noted (Bisson *et al.*, 1976; Miura *et al.*, 1998). Studies on genetic factors regulating fertility/fecundity and response to environmental agents have been at best extremely limited. Recent studies suggest that inter-individual differences in expression of isoforms of certain enzymes underlie the response to airborne environmental toxicants (e.g. GST and *N*-acetyltransferase versus effects of polycyclic aromatic hydrocarbons; Sram *et al.*, 1996) and may contribute to the production of endometriosis (Baranova *et al.*, 1997) or an increase in spontaneous abortions (Wang *et al.*, 1998). In contrast, we do not know why occupational exposure to suspected reproductive toxicants appears to have a greater effect on male fecundity than do female occupational exposures (Spinelli *et al.*, 1997), or why men are more sensitive than women to environmental exposure to metals (Staessen *et al.*, 1991; Dickman *et al.*, 1998) or organic toxicants (Welch *et al.*, 1988; Schrader, 1997).

Clearly, there is much to learn about the factors which regulate susceptibility. We believe that analysis of infertility patients offers a unique opportunity to address these factors. Our data suggest that infertility patients may be predisposed to and may respond more strongly to environmental metal exposures than the population in general.

Ion channels and response to environmental metal exposures

Acrosome exocytosis induced by sperm–zona binding and required for sperm penetration through the zona pellucida involves sequential activation of sperm head delayed (outward) rectifier voltage-gated potassium (K^+) channel (VGKC) and L-type voltage-dependent Ca^{2+} (VDCC) ion channels (L-VDCC) (Benoff, 1999). Distinct binding sites for Pb^{2+} , Cd^{2+} and other metal ions appear to regulate ion permeation (Leinders *et al.*, 1992). Each type of channel is composed of multiple subunits. When expressed *in vitro*, α subunits possess all characteristics necessary to form channel pores (Catterall, 1995; Benoff, 1998).

The α subunit of the typical VGKC consists of six putative transmembrane segments designated S1–S6 (Catterall, 1995). Transmembrane segment S4 is the voltage sensor. The regions between transmembrane segments S5 and S6 contain the ion conducting pore and binding sites for channel blockers and metals ions (Shieh and Kirsch, 1994; Catterall, 1995; Christie, 1995; Doyle *et al.*, 1998). Different isoforms result from sequence changes in the ion conduction pore (Grissmer *et al.*, 1994). Thus, genetic polymorphisms in these ion channels can regulate resistance or susceptibility to environmental metal exposures.

The α -1 subunits of VDCC are related to those of VGKC, being derived from a common ancestor (Catterall, 1995). VDCC α -1 subunits are composed of four repeats (domains I–IV) of the basic structural unit of the VGKC α subunit (S1–S6) (Catterall, 1995; MacKinnon, 1995). Multiple L-VDCC α -1 isoforms (e.g. α -1A, α -1B, α -1C, α -1D, α -1E) are generated by transcription of different genes and by alternate splicing of the primary transcript from each gene (Benoff, 1998). The α -1C subunit forms the pore

of the L-VDCC expressed in mammalian testis and spermatozoa (Benoff, 1998,1999; Goodwin *et al.*, 1999a,b). This subunit contains the binding sites for a variety of pharmacological agents, including dihydropyridines which are prescribed for hypertension control and which can produce a reversible infertile state (Benoff *et al.*, 1994; Hershlag *et al.*, 1995). Ion selectivity (e.g. Ca^{2+} versus Na^+) is conferred by single amino acid changes in the extracellular mouth of the pore and/or the pore wall itself (Heinemann *et al.*, 1992). By analogy with Na^+ channels, sensitivity or resistance to metals ions will also be conferred by single amino acid substitutions (i.e. Backx *et al.*, 1992; Satin *et al.*, 1992).

Genetic polymorphisms in these ion channels expressed in somatic cells have been associated with the generation of diseases. For example, a study of CCG repeat polymorphisms in human brain cDNA (targeted to develop markers for neuro-psychiatric disorders including autism) amplified a partial clone of BCNG-1 (Kleiderlein *et al.*, 1998). BCNG-1 has been identified as encoding a voltage-gated potassium (K^+) channel (VGKC) with properties indistinguishable from brain pacemaker channels (Santoro *et al.*, 1998). If BCNG-1 shows the sensitivity to Pb^{2+} of other voltage-gated K^+ channels (Kiss and Osipenko, 1994), its polymorphic expression may provide a mechanism for the variability confounding neuro-psychiatric effects of paediatric Pb^{2+} exposure (Needleman, 1988).

With regard to VDCC, alternate splicing in the carboxy terminus of the α -1A subunit is linked to a severe form of human ataxia (Zhuchenko *et al.*, 1997). Sequence changes, including one in transmembrane segment IIIS2, of the α -1A result in familial hemiplegic migraine and episodic ataxia type-2 where patients exhibit migraine-like symptoms (Ophoff *et al.*, 1996). These findings are of particular importance given that we have identified multiple splice variants of the human α -1C subunit that are expressed in testis and spermatozoa. The alternatively spliced regions include the amino terminus and transmembrane segments IS6, IIIS2 and IVS3 (see section on varicocele-associated infertility below).

Potential models for metal effects mediated by ion channels

We now present evidence that the interaction between environmental exposure to Cd^{2+} and L-VDCC results in infertility in the presence of a varicocele. We also report that many cases of 'unexplained' infertility can be attributed to the interaction of VGKC and environmental Pb^{2+} exposures.

Varicocele-associated infertility

Varicocele-associated infertility is widespread: ~40% of males from infertile couples present with varicocele (Greenberg, 1977; Goldstein *et al.*, 1992; WHO, 1992; Schlesinger *et al.* 1994). The mechanism(s) underlying varicocele-associated infertility, however, are poorly characterized. The most widely accepted explanation for the pathophysiology of varicocele in male infertility is abnormally elevated testicular temperature due to impaired heat transfer by the scrotum and/or changes in testicular blood flow (Comhaire, 1991). Although men with varicocele exhibit higher mean scrotal temperatures, there is a large overlap with the range of scrotal temperatures in fertile men (Mieusset and

Bujan, 1995). More importantly, only 13% of men with varicocele are infertile (Kursh, 1987; Sylora and Pryor, 1994), and, although varicocele repair has been documented to reduce testicular temperature (Agger, 1971; Yamaguchi *et al.*, 1989), only 1/3 of infertile men with varicocele will experience a return of fecundity following varicocele correction (Schlesinger *et al.*, 1994). These findings suggest that varicocele may not be a primary cause of infertility and that it is the interaction of varicocele with other as yet unidentified factors that produces the infertile state (Peng *et al.*, 1990; Gentile and Cockett, 1991; Mieusset and Bujan, 1995).

We suggest that one such factor is the concentration of Cd2+ in the male reproductive tract (Benoff, 1997; Benoff *et al.*, 1997, 1998a). In animal varicocele models, exposure to components of cigarette smoke increases impairment of spermatogenesis. Although there is some controversy as to whether smoking reduces semen quality (Goverde *et al.*, 1995; Hughes and Brennan, 1996) or reduces fertility (Vine, 1996) in all men of reproductive age, it is generally agreed that men with marginal semen quality may be made infertile by smoking (see discussion in section on examination of the effects of environmental exposure to metal ions above). Oligozoospermia among men with varicocele who also smoke is: (i) 10-fold more frequent than among other men with varicocele and (ii) five-fold more frequent than among smokers without varicocele (Klaiber *et al.*, 1987).

Our data indicate that the concentration of Cd2+ in seminal plasma from infertile men with varicocele is markedly increased, compared with fertile men with varicocele (Figure 1) or men with other forms of infertility (e.g. see Figure 2). Recent findings indicate that Cd2+ concentrations are similarly increased in the testis of infertile men with varicocele (Figure 1; Benoff *et al.*, 1999b). After varicocele repair, pregnancy by coitus was observed only in those men whose Cd2+ concentrations were at the lower end of the range (Benoff *et al.*, 1998a). These findings suggest that measurements of seminal plasma Cd2+ concentrations may serve as a biomarker for the outcome of varicocele repair.

Men with secondary infertility and varicocele present with seminal plasma Cd2+ concentrations intermediate between those of fertile men and men with primary varicocele-associated infertility (Benoff *et al.*, 1999b), suggesting that the deleterious effects of Cd2+ are time and concentration dependent. Blood plasma Cd2+ concentrations did not, however, differ significantly between infertile and fertile men with varicocele (Fig. 1). These data emphasize that the choice of variable to be analysed influences whether or not an association is detected between an environmental exposure and production of the infertile state.

It is known that infertile men with varicocele are typically oligozoospermic. The sperm populations from these men have increased tapering head forms ('stress pattern'), reduced zona pellucida binding ability and an acrosome reaction insufficiency (Rogers *et al.*, 1985; Naftulin *et al.*, 1991; Vigil *et al.*, 1994; Benoff *et al.*, 1995a, 1997; El Mulla *et al.*, 1995; Benoff, 1997). These defects can clearly result from an interaction between the observed increase in testicular Cd2+ and actin. Cd2+ exposure results in depolymerization of actin filaments and a net decrease in actin monomer protein (Wang *et al.*, 1996). In somatic cells, actin loss increases apoptosis (Russo *et al.*, 1982; Tsukidate *et al.*,

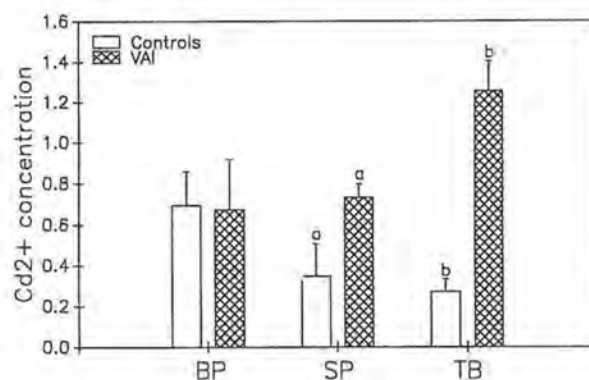


Figure 1. Comparison of the cadmium (Cd2+) content in blood plasma (BP), seminal plasma (SP) and testis biopsies (TB) of infertile men with varicocele (VAI) and men without varicocele (controls). Cd2+ concentrations were determined by graphite furnace atomic absorption spectroscopy (Benoff, 1997; Benoff *et al.*, 1997, 1998a, 1999b; Hurley *et al.*, 1997). The bars in the figure indicate the BP and SP Cd2+ content in $\mu\text{g/l}$ and in testis biopsies (obtained by percutaneous needle aspiration; Marmar, 1998) in ng/mg dry weight. The concentrations of Cd2+ in blood plasma from male partners without varicocele from couples seeking fertility evaluation ($n = 66$) and from VAI males ($n = 14$) were similar. In contrast, SP Cd2+ concentrations were significantly elevated in men with VAI ($n = 14$), compared with fertile men with varicocele ($n = 4$) (a, $P < 0.0001$; t -test). Similarly, TB Cd2+ levels were higher in men with VAI [$n = 8$; Johnsen score (Johnsen, 1970) = 8–9] than in TB from men with non-obstructive azoospermia ($n = 4$; Johnsen score = 6) (b, $P < 0.03$; t -test).

1993) (which could lead to oligozoospermia in infertile men with varicocele) and decreases the ability to undergo exocytosis (Muallem *et al.*, 1995) (which requires both actin polymerization and depolymerization, as in the acrosome reaction; Rogers *et al.*, 1989; Spungin *et al.*, 1995; Benoff *et al.*, 1996; Benoff, 1999).

Preliminary examination of testis biopsies from infertile men with varicocele suggests that this indeed is the case. Actin immunoreactivity was decreased and apoptosis, which normally occurs at low concentrations during spermatogenesis (Furuchi *et al.*, 1996; Tesarik *et al.*, 1998) was increased, compared with biopsies from men without varicocele (Benoff *et al.*, 1999b). These data are consistent with findings in animal models wherein in-vivo administration of Cd2+ induced apoptosis in testicular tissue (Xu *et al.*, 1996; Yan *et al.*, 1997).

In-vitro modelling experiments support our in-vivo findings (Benoff, 1997; Benoff *et al.*, 1997). Cd2+ exposure changed fertile donor sperm protein expression (e.g. loss of sperm head actin, reduced percentages of spermatozoa expressing surface mannose and non-nuclear progesterone receptors) and function (e.g. acrosome reaction insufficiency) to mimic varicocele in a dose-response fashion, albeit at Cd2+ concentrations >30-fold higher than in seminal plasma from infertile men with varicocele. After fertile donor spermatozoa were briefly exposed to higher temperatures, lower Cd2+ concentrations produced more drastic sperm function changes, indicating that temperature and Cd2+ act synergistically, as in cryptorchid animal models (Chatterjee and Ray, 1972; Fende and Niewenhuis, 1977).

Increased testicular Cd2+ concentrations are probably derived from the increased transvascular fluid exchange which occurs with varicocele (Shafik and Bedeir, 1980; Sweeney *et al.*, 1995).

This means that there is the potential for more of the Cd^{2+} in serum to enter the testis. As there is no active pump to remove this Cd^{2+} (Gunn *et al.*, 1961), testicular Cd^{2+} concentrations would be elevated over time. Results from autometallography (Benoff, 1998, 1999; Benoff *et al.*, 1999b) indicate that this Cd^{2+} can enter spermatogenic cells via L-VDCC (Kiss and Osipenko, 1994; Atar *et al.*, 1995), which are expressed in all cells within the germinal epithelium (Goodwin *et al.*, 1998). The multiple isoforms of the testis-specific L-VDCC which have been identified result from alternate splicing of a primary transcript from a single gene (Benoff, 1998). Preliminary findings suggest that each man expresses only one L-VDCC splice variant in his ejaculated spermatozoa (Goodwin *et al.*, 1999a,b). The Cd^{2+} sensitivity or resistance of L-VDCC is conferred by the amino acid sequence within a short segment of the protein (domain I, transmembrane segment S6; e.g. Backx *et al.*, 1992). This region is alternatively spliced in the testis-specific L-VDCC and a series of amino acid substitutions within this region have also been observed (Benoff, 1999). Thus, our working hypothesis is that infertility in these men results from the interaction of three factors: (i) the presence of a varicocele; (ii) an increase in testicular Cd^{2+} concentrations; and (iii) the expression of particular L-VDCC isoforms.

Lead and unexplained infertility

We have been prospectively evaluating men from infertile couples undergoing their first IVF cycle in order to identify biomarkers which predict fertilization outcome and which are not related to sperm count, motility, viability or morphology (Benoff *et al.*, 1999a). An unexpected finding was that >40% of these males, who were not exposed to Pb^{2+} in their places of work and who did not smoke cigarettes, exhibited blood and seminal plasma Pb^{2+} concentrations that were above the permissible limit in men occupationally exposed to Pb^{2+} (Benoff *et al.*, 1998b). Both blood and seminal plasma Pb^{2+} concentrations in these men were inversely correlated with the rate of fertilization of metaphase II oocytes in IVF (Benoff *et al.*, 1998b; Benoff, 1999; see Figure 2). The negative effect of increased Pb^{2+} on IVF outcome was attributable to altered sperm function, i.e. increased spontaneous acrosome loss and a decreased ability to undergo an acrosome reaction induced by exposure of capacitated spermatozoa to progesterone or model zona ligands containing mannose. The progesterone-stimulated acrosome reaction was the parameter most severely affected by Pb^{2+} . Mannose receptors and non-nuclear progesterone receptors are co-expressed on a subpopulation of motile human spermatozoa (Benoff *et al.*, 1995c) and the response to progesterone determines whether or not human spermatozoa undergo an acrosome reaction after zona binding and fertilize human oocytes (Jacob *et al.*, 1998c).

Results from cDNA cloning and in-vitro modelling studies employing K^+ channel inhibitors provided evidence that the effects of progesterone on the human sperm acrosome reaction were mediated by a delayed rectifier VGKC (Jacob *et al.*, 1997a,b, 1998a,b) and that this VGKC is upstream of the L-VDCC which regulates the sustained Ca^{2+} influx required for the AR (Benoff, 1999). K^+ channels in somatic tissues can be inhibited by Pb^{2+} (Kiss and Osipenko, 1994) and environmental Pb^{2+} exposures have been reported to alter K^+ transport by red blood cells (Hajem *et al.*, 1990).

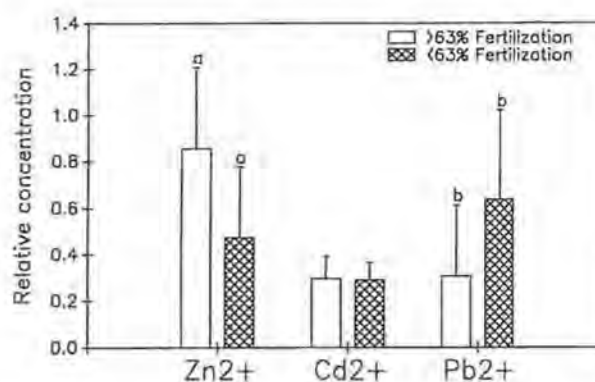


Figure 2. Comparison of the concentrations of zinc (Zn^{2+}), cadmium (Cd^{2+}) and lead (Pb^{2+}) in seminal plasma from men exhibiting high rates of fertilization following conventional IVF and men exhibiting reduced or failed fertilization. In a prospective analysis, we evaluated the concentrations of metals in seminal plasma (SP) from men from consecutive couples undergoing their first cycle of conventional IVF ($n = 96$). The number of spermatozoa used in each inseminate was normalized on the basis of acrosomal morphology, so that at least 25 000 spermatozoa with an oval head with a well-defined acrosome covering >50% of the head were inseminated per oocyte cultured in 1 ml of medium (Benoff *et al.*, 1999a). The mean fertilization for this study population was $85.2 \pm 11.1\%$ (Jacob *et al.*, 1998c; Benoff *et al.*, 1999a). Cases of 'reduced' fertilization were defined as exhibiting fertilization rates >2 SD below the mean, e.g. <63%. Cases of 'high' fertilization were defined as exhibiting fertilization rates >63%. SP metal concentrations were determined by graphite furnace atomic absorption spectroscopy (Benoff, 1997; Benoff *et al.*, 1997, 1998b; Hurley *et al.*, 1997; S. Benoff and I.R. Hurley, unpublished). SP Zn^{2+} concentrations are presented as millimolar concentrations while SP Cd^{2+} and SP Pb^{2+} concentrations are reported as $\mu\text{g/l}$. Although SP Cd^{2+} did not differ between the two groups, SP Zn^{2+} was markedly decreased (a, $P < 0.0001$; t -test) and SP Pb^{2+} was significantly increased (b, $P < 0.0002$; t -test) in cases of reduced fertilization, compared with cases with high fertilization rates.

Sperm surface VGKC were mapped using a biotinylated charybdotoxin (CBTx) probe (Figure 3a,b). Of spermatozoa from fertile donors ~38% exhibited head-directed CBTx binding. Sites of exogenous Pb^{2+} entry into the human sperm head were similarly localized by autometallography (Figure 3c,d). The same percentages of spermatozoa that bound CBTx also bound Pb^{2+} , providing strong evidence that a VGKC capable of transporting Pb^{2+} is expressed on the human sperm head. Preliminary results from in-vitro modelling studies confirmed these findings (Figure 4). CBTx strongly inhibited the increase in spontaneous acrosome loss which normally results from Pb^{2+} exposure. These data are consistent with findings in animal models that in-vivo Pb^{2+} exposure causes premature acrosome reactions (Johansson, 1989) and provide strong support for our hypothesis that Pb^{2+} action to reduce human sperm fertilizing potential is mediated by a sperm head VGKC.

Analysis of the effects of the K^+ channel inhibitors CBTx, tetraethylammonium ion (TEA), and 4-aminopyridine (4AP) on progesterone-stimulated acrosome loss by fertile donor spermatozoa provided evidence for inter-male variability in VGKC isoform expression (Benoff, 1999). The existence of multiple VGKC isoforms would help to explain why two out of 53 men who exhibited high rates of fertilization had seminal plasma Pb^{2+} concentrations that were >10 $\mu\text{g/dl}$ (the upper limit of the range of blood Pb^{2+} concentrations in unexposed men) and three

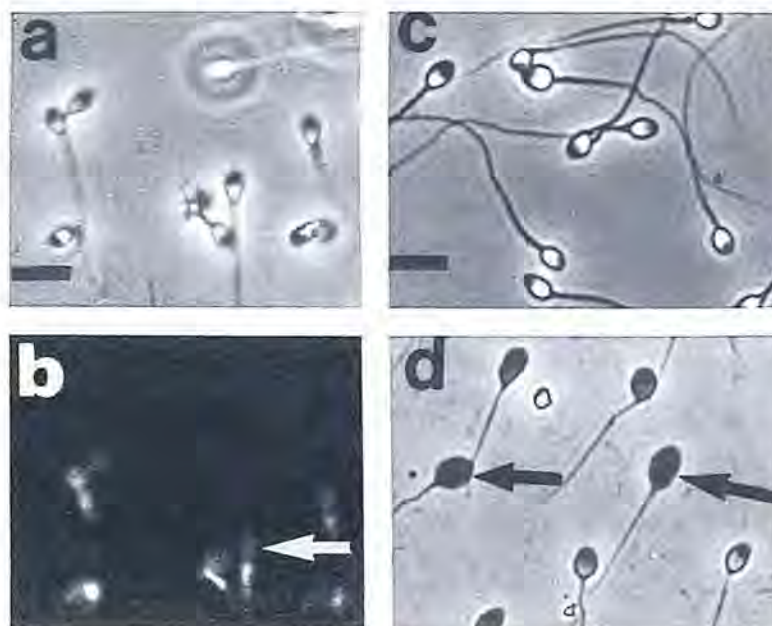


Figure 3. Voltage-gated potassium channels and sites of exogenous lead entry co-localize on the human sperm head surface. Specimens were viewed at $\times 400$ original magnification with an Olympus microscope (Olympus Corp., Lake Success, NY, USA) and photographed at $\times 600$ on 35 mm/400 ASA black and white film (Eastman Kodak Co, Rochester, NY, USA) with automatic exposure for phase-contrast images (a, c, d) and 50 s exposure for the epifluorescence image (b). (a, b) A minimally biotinylated charybdotoxin probe was prepared by modifying the protocols supplied with commercial reagents (Pierce, Rockford, IL; Bio Rad Laboratories, Hercules, CA, USA) (A.Jacob, I.R. Hurley and S.Benoff, unpublished observations). Motile spermatozoa from fertile donors were surface labelled with the biotinylated charybdotoxin and sperm-bound charybdotoxin was visualized by indirect immunocytochemistry using polyclonal antibodies to biotin prepared in rabbit (Enzo Diagnostics, New York, NY, USA). (a) Paired phase-contrast and (b) epifluorescence images are shown. The arrow in the figure points to charybdotoxin binding over the entire sperm head surface. Scale bar = 12.5 μm . (c, d) Motile spermatozoa from fertile donors were incubated overnight in capacitation media supplemented with 2.5 $\mu\text{mol/l}$ lead (Pb^{2+}). Sperm-bound Pb^{2+} as localized by autometallography (Stoltenberg *et al.*, 1997a,b). Pb^{2+} bound over the entire sperm head is visualized as black silver deposits (d, arrow) after conversion of Pb^{2+} to Pb sulphide and physical development with silver lactate. Black silver deposits are not detected in control aliquots (c) exposed to Pb^{2+} but not treated with sodium sulphide prior to physical development. Scale bar = 12.5 μm .

out of 21 men who exhibited reduced or failed fertilization had Pb^{2+} concentrations $<10 \mu\text{g/dl}$. Further studies are now in progress to identify the specific structural variations in the human sperm VGKC α subunit which regulate sensitivity to Pb^{2+} .

Conclusions

We are left with a puzzle. The clinical data presented above show that two groups of infertile men have abnormally high metal concentrations in seminal plasma, without any obvious recent high exposure to these toxicants. The in-vitro studies and the molecular genetics provide, in outline, a plausible mechanism showing how these toxicants could produce the characteristic clinical symptoms of each type of infertility. However, this does not explain the aetiology of high metal concentrations. Both Cd^{2+} (characteristic of the varicocele-related infertile group) and Pb^{2+} (high spontaneous acrosome reaction group) are metals with a low clearance rate. It is possible that members of both groups had some unrecorded high exposure to these metals in the past, and their blood and seminal plasma concentrations are equilibrium concentrations reflective of normal clearance. This is hard to credit, as an exposure large enough to give the concentrations we observed after an uncertain number of years of the initial exposure is likely to have exhibited clinically significant symptoms at the time of that exposure. Another possibility is that the individuals with high metals concentrations have some other condition which

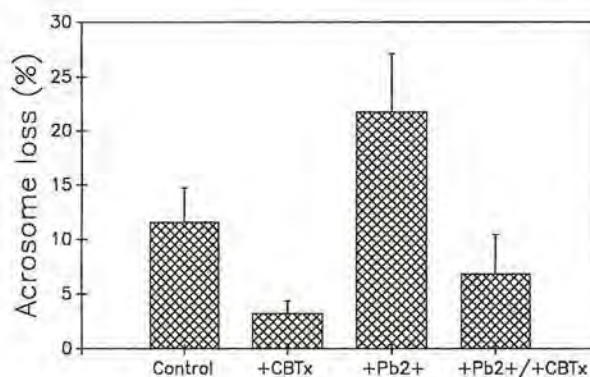


Figure 4. Opposing effects of lead (Pb^{2+}) and charybdotoxin (CBTx) on the spontaneous acrosome reaction. Motile sperm populations from fertile donors ($n = 5$) were divided and exposed to 50 nmol/l CBTx or 2.5 $\mu\text{mol/l}$ Pb^{2+} , alone or in combination, and compared with aliquots not exposed to either agent (control). Spontaneous acrosome loss was assessed following reaction with rhodamine-labelled *Pisum sativum* agglutinin, which binds to acrosome content (Benoff *et al.*, 1995b). Pb^{2+} exposure significantly increased acrosome loss ($P < 0.007$). CBTx decreased both the level of control acrosome loss ($P < 0.005$) and that observed following Pb^{2+} exposure ($P < 0.006$). The percentages of spermatozoa undergoing an acrosome reaction in the presence of both Pb^{2+} and CBTx were similar to that in the presence of CBTx alone (not significant).

predisposes them to accumulate high amounts of toxicant from exposures no higher than those most of us encounter. This

condition may be dietary, related to intake of some other metals or minerals which alter the bio-availability of toxicants. Alternatively, these conditions may be genetic, involving isoforms of proteins involved in transport, membrane passage or storage of metals. If genetic predisposition does indeed create susceptibility subgroups with significantly lower tolerance of toxicant exposures, it may be necessary to reconsider the standards embodied in current legislation.

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Memorandum

Date: May 7, 2001

From: Roy M. Fleming, Sc.D., Director, Research Grants Program *RMF*
Office of Extramural Programs, NIOSH, D30

Subject: Final Report Submitted for Entry into NTIS for Grant 5 R03 OH003584-02.

To: William D. Bennett
Data Systems Team, Information Resources Branch, EID, NIOSH, P03/C18

The attached final report has been received from the principal investigator on the subject NIOSH grant. If this document is forwarded to the National Technical Information Service, please let us know when a document number is known so that we can inform anyone who inquires about this final report.

Any publications that are included with this report are highlighted on the list below.

Attachment

cc: Sherri Diana, EID, P03/C13

List of Publications

Jacob A, Hurley IR, Goodwin LO, Cooper GW, Benoff S: Molecular Characterization of A Voltage-Gated Potassium Channel Expressed in Rat Testis. Mol Hum Reprod 6:303-313, 2000

Benoff S, Jacob A, Hurley IR: Male Infertility and Environmental Exposure to Lead and Cadmium. Hum Reprod Update 6:107-121, 2000

Benoff S, Jacob A: Human Sperm Non-Nuclear Progesterone Receptor: A Novel Marker for Fertilization Outcome. Society for Male Reproduction and Urology Post-Graduate Course, Laboratory Methods: Current Methods and a Look Toward the Future, Birmingham: American Society for Reproductive Medicine, 1999

Title: Sperm Progesterone Receptor, K⁺ Channel & Pb Exposure
Investigator: Asha Jacob, Ph.D.
Affiliation: North Shore University Hospital
City & State: Manhasset, NY
Telephone: (516) 562-1049
Award Number: 5 R03 OH003584-02
Start & End Date: 7/1/1998–6/30/2000
Total Project Cost: \$79,440
Program Area: Exposure Assessment Methods
Key Words:

Abstract:

The objective of our lab is to identify biomarker which predict fertilization outcome in IVF. Conventional markers such as motility, viability or morphology failed to correlate with fertility potential. Our recent prospective study on infertile couples undergoing their first cycle of IVF demonstrated that >40% of these males exhibited high levels of Pb²⁺ in their seminal plasma. These high levels of Pb²⁺ inversely affected sperm function (i.e. increased spontaneous acrosome loss and decreased ability to undergo an acrosome reaction induced by exposure to progesterone (PSAR) or zona ligands (i.e. mannose). The PSAR was the parameter mostly affected by Pb²⁺. Our preliminary studies on K⁺ channel inhibitors suggested that the effects of progesterone on the human sperm acrosome reaction are mediated by a K⁺ channel. Thus, we hypothesized that sensitivity to Pb²⁺ exposure alters human sperm acrosome reaction by the interaction with a K⁺ channel. To test this hypothesis, we characterized the human sperm K⁺ channel. We examined the ability of human sperm to undergo PSAR in the presence of specific K⁺ channel inhibitors. Our data clearly showed that sperm from men who respond to progesterone exhibited an average of 30-40% inhibition of the PSAR in the presence of K⁺ channel inhibitors. These inhibitors similarly affected spontaneous acrosome reaction SAR. Interestingly, Pb²⁺ induced SAR was inhibited by these channel blockers. We localized human sperm surface K⁺ channel binding sites using biotin-coupled CBTX, a specific K⁺ channel blocker. Independent to our observation, other members in the lab showed that Pb²⁺ entry co-localized with K⁺ channel binding sites on human sperm. Thus, biochemical analyses confirmed our prior preliminary findings that a K⁺ channel mediated the effects of progesterone on the human sperm acrosome reaction. Thus, Pb²⁺ associated alternations in human sperm function could be in part mediated by a K⁺ channel. We examined by RT-PCR the expression of KCNA3 in human sperm. For these analyses, we have designed primers from the S4 and S6 region of KCNA3 that contains the ion conduction pore of the channel and binding sites for all K⁺ channel inhibitors used in the biochemical analyses described above. RT-PCR analysis on human sperm using these primers generated specific PCR products generated specific PCR products that were 98% homologous to KCNA3. Our data suggest that the presence of these K⁺ channel mRNA positively correlated with the ability of human sperm to mount a PSAR. We now identified two individuals whose sperm fail to express the K⁺ channel transcript and do not undergo PSAR. Last, we examined by RT-in situ PCR analyses, the expression pattern of these K⁺ channel transcripts in human sperm. These channel transcripts were expressed over the entire human sperm head in 20-30% of

sperm. Thus, these data provide strong evidence for the presence of either KCNA3 or KCNA3 related K⁺ channels in human sperm.

Our prior studies on the rat testis K⁺ channel suggest that more than one isoform of K⁺ channel is expressed in rat testis. Our preliminary analyses on human sperm also suggest the presence of different isoforms of K⁺ channels.

Publications

Jacob A, Hurley IR, Goodwin LO, Cooper GW, Benoff S: Molecular Characterization of A Voltage-Gated Potassium Channel Expressed in Rat Testis. *Mol Hum Reprod* 6:303-313, 2000

Benoff S, Jacob A, Hurley IR: Male Infertility and Environmental Exposure to Lead and Cadmium. *Hum Reprod Update* 6:107-121, 2000

Benoff S, Jacob A: Human Sperm Non-Nuclear Progesterone Receptor: A Novel Marker for Fertilization Outcome. Society for Male Reproduction and Urology Post-Graduate Course, Laboratory Methods: Current Methods and a Look Toward the Future, Birmingham: American Society for Reproductive Medicine, 1999