

Link between low-dose environmentally relevant cadmium exposures and asthenozoospermia in a rat model

Susan Benoff, Ph.D.,^{a,b,c} Karen Auborn, Ph.D.,^{d,e} Joel L. Marmar, M.D.,^f and Ian R. Hurley, Ph.D.^a

^a Fertility Research Laboratories, The Feinstein Institute for Medical Research, Manhasset, New York; ^b Department of Obstetrics and Gynecology, North Shore University Hospital, North Shore-Long Island Jewish Health System, Manhasset, New York; ^c Departments of Obstetrics & Gynecology and Cell Biology, New York University School of Medicine, New York, New York; ^d Phytochemical Research Laboratory, The Feinstein Institute for Medical Research, Manhasset, New York; ^e Department of Otolaryngology, Long Island Jewish Medical Center, The Long Island Campus of Albert Einstein College of Medicine, New Hyde Park, New York; and ^f Division of Urology, Department of Surgery, Robert Wood Johnson Medical School, Camden, New Jersey

Objective: To define the mechanism(s) underlying an association between asthenozoospermia and elevated blood, seminal plasma, and testicular cadmium levels in infertile human males using a rat model of environmentally relevant cadmium exposures.

Setting: University medical center andrology research laboratory.

Animal(s): Male Wistar rats (n = 60), documented to be sensitive to the testicular effects of cadmium.

Intervention(s): Rats were given ad libitum access to water supplemented with 14% sucrose and 0 mg/L, 5 mg/L, 50 mg/L, or 100 mg/L cadmium for 1, 4, or 8 weeks beginning at puberty.

Main Outcome Measure(s): Testicular cadmium levels were determined by atomic absorption, cauda epididymal sperm motility by visual inspection, and testicular gene expression by DNA microarray hybridization.

Result(s): Chronic, low-dose cadmium exposures produced a time- and dose-dependent reduction in sperm motility. Transcription of genes regulated by calcium and expression of L-type voltage-dependent calcium channel mRNA splicing variants were altered by cadmium exposure. Expression of calcium binding proteins involved in modulation of sperm motility was unaffected.

Conclusion(s): A causal relationship between elevated testicular cadmium and asthenozoospermia was identified. Aberrant sperm motility was correlated with altered expression of L-type voltage-dependent calcium channel isoforms found on the sperm tail, which regulate calcium and cadmium influx. (Fertil Steril® 2008;89:e73–9. ©2008 by American Society for Reproductive Medicine.)

Key Words: Cadmium, asthenozoospermia, sperm tail, calcium channels

Cadmium is one of the most toxic metals, with no known beneficial physiologic role. Cadmium represents a serious health hazard to humans (1, 2) because of its long half-life (e.g., ~20–30 years in humans) and low rate of excretion (<1–2 µg/day) (3–5), its storage in the male reproductive tract (6, 7), and its carcinogenic potential (8).

More cadmium is released into the environment (25,000–30,000 tons annually) than most other carcinogenic toxicants. The 2003 US Comprehensive Response Compensation and Liability Act's Priority List of Hazardous Substances recognizes cadmium by listing it at number seven, above any pesticide. Cadmium release arises from use of cadmium metal in batteries and plating, and of cadmium

compounds in computers, pigments, and glazes (9). However, most environmental cadmium exposures are not associated with waste sites (10). Cadmium is released into water as a by-product of smelting, into air by combustion of coal and oil, and into soils as impurities (9). Humans accumulate cadmium primarily by consuming foods: leafy vegetables contain 10–40 µg/kg cadmium; organ meats and fresh water fishes contain more (10, 11). Adsorption of cadmium through the gut is relatively inefficient. Daily adult dosage from food is 1–5 µg/day. Lung absorption is more efficient; smoking a pack of cigarettes a day effectively doubles the daily dose (9). Human air exposures from fossil fuel particulates are much smaller because cadmium is not directly delivered to lungs. Nonetheless, airborne cadmium exposures are ubiquitous. Cadmium is present in aerosol sprays and cleaning products used in most American homes as a potentially very dangerous added component. Given these observations, it is not unexpected that cadmium was detected in the majority of blood and urine samples collected from males participating in the National Health and Nutrition Examination Survey (12).

Our interest in the role of cadmium in the modulation of male reproductive health stems from our observations that cadmium is elevated in seminal plasma from infertile men

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Reprint requests: Susan Benoff, Ph.D., The Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, New York 11030 (FAX: 516-562-1022; E-mail: Sbenoff@nshs.edu).

presenting with asthenozoospermia (13). Some surveys by others of human blood and seminal plasma also find high cadmium concentrations are associated with poor sperm motility in nonsmokers (14–17) and in smokers (15, 18–23). This not a uniform finding (24, 25), possibly because of levels of confounders (21) or human genetic variation among the study population (9). However, animal studies demonstrated a time- and dose-dependent reduction in sperm motility following cadmium exposures (26–29), identifying a causal relationship that supported the correlative data cited that was obtained in humans. These findings have stimulated the current research for several reasons.

Cadmium is a known competitor of calcium (30, 31), and calcium homeostasis is central to sperm motility regulation. Normal sperm motility is primarily controlled by cAMP/PKA and calcium signaling pathways, which probably interact by controlling, respectively, phosphorylation and dephosphorylation of proteins in the sperm tail (32–36). The calcium signaling pathway involves calcium influx through ion channels on the sperm tail plasma membrane, activation of calmodulin and calcium/calmodulin-dependent serine/threonine protein kinase IV (CaMK IV) (37), activation of additional calcium channels, and finally, activation of calcineurin, a calcium/calmodulin-dependent serine/threonine phosphatase. Thus, there are multiple targets for cadmium action. In addition, several other mechanisms related to cadmium action on sperm tail proteins have not yet been investigated. For example, cadmium could act indirectly on motility through modulation of gene transcription (38–40) and/or pre-mRNA splicing (41–43). Simply put, there is still a lot to learn on this subject, and we believe that the tools are available for meaningful investigations.

As part of some of our studies of male infertility, we have observed that many men with asthenozoospermia also have elevated levels of cadmium in their testes (44–46). Curiously, these men were not occupationally exposed to cadmium and did not smoke cigarettes. Therefore, in the current pilot study we intend to expand our genetic and molecular knowledge of the relationship between cadmium and poor sperm motility, and we have employed a cadmium-sensitive rat model to examine the effects of low-dose chronic cadmium exposures on testicular expression of genes regulated by calcium, of calcium binding proteins, and of splice variants of calcium-permeable ion channels expressed on the sperm tail.

MATERIALS AND METHODS

Cadmium Solutions

Cadmium solutions were prepared using cadmium chloride ordered from Alfa Aesar (Cat. No. 12373 ACS; $\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$), where 1 g of cadmium equals 2.03 g of 81% pure CdCl_2 . The initial stock was prepared as 4g/L cadmium. Serial dilutions were prepared to yield stocks of 200 mg/L, 100 mg/L, and 10 mg/L. Each of these stocks was diluted 1:1 with 28% sterile sucrose to yield the working

solutions containing 100 mg/L, 50 mg/L, and 5 mg/L cadmium in 14% sucrose.

Before animal exposure, the cadmium content of all drinking water solutions was assayed and verified by graphite furnace atomic absorption spectroscopy (GFAAS) using our published protocols (13, 45).

Animal Model

The protocols employed were approved by the Institutional Animal Care and Use Committees of the North Shore-Long Island Jewish Health System. Animals were maintained in accordance with the standards set forth in the Animal Welfare Act. All studies were performed using Wistar rats. This strain of rats is sensitive to the testicular effects of cadmium (47), and chronic low-dose exposures produce persistent testicular lesions and infertility (48).

Four-week-old male Wistar Hanover rats were purchased from Charles River Laboratories, Inc. (Wilmington, MA) and allowed to acclimate for 1 week in the Feinstein Institute's Center for Comparative Physiology. Rats were housed two per cage. Cadmium exposures were initiated at puberty (5 weeks of age; the time at which testicular blood vessels and spermatogenesis become sensitive to the toxic effects of cadmium) (49, 50) with exposure durations of 1 week, 4 weeks, and 8 weeks. Cadmium was administered orally, thus paralleling human exposures to cadmium contamination by food or water.

Rats were given ad libitum access to a nutritionally complete solid diet without added cadmium in combination with either: [1] deionized water with 14% sucrose but no added cadmium (51), [2] deionized water 14% sucrose and 5 mg/L cadmium as CdCl_2 (environmentally realistic low dose) (52), [3] deionized water with 14% sucrose and 50 mg/L cadmium as CaCl_2 (environmentally realistic intermediate dose) (52, 53), or [4] deionized water with 14% sucrose and 100 mg/L cadmium as CaCl_2 (environmentally realistic high dose) (52–54). Some rats may drink less because of the taste of cadmium (55), so 14% sucrose was added to all drinking water to make it a more desirable drink (51).

Animals were weighed twice per week. Water consumption by weight was measured over a 24-hour period twice per week. Based on measured water consumption, the effective doses of cadmium received were, respectively, 0 mg/kg, 1.6 mg/kg, 6.4 mg/kg, and 12.6 mg/kg body weight/day, which represent low to moderate environmental exposures (56). Under these conditions, cadmium (as detected by GFAAS) (45) accumulated in the Wistar rat testes in a time- and dose-dependent manner, approaching levels observed in infertile human population studied (>0.4 ng/mg dry wt) (45, 46).

Analysis of Sperm Motility

Sperm were isolated from dissected epididymes at time of sacrifice following published protocols (57). Following

incubation at 37°C for 10–15 minutes, a drop of the sperm suspension was placed on a prewarmed hemocytometer. Sperm motility was determined in triplet counts of 100 sperm each, noting the percentage of sperm moving (including those that were twitching but not exhibiting forward motility).

Statistical analyses were performed with SigmaStat v 3.0 software package (SSPS, Inc., Chicago, IL). Statistical significance was set at $P < .05$.

RNA Isolation and DNA Microarray Hybridization

In this pilot study, gene expression was examined in the left testes at 1 week and at 8 weeks of cadmium exposures in the control (no cadmium) and 100 mg/L cadmium-exposed rats using five animals per time and dose.

RNA was extracted from testis slices using reagents from Qiagen (RNeasy Mini Kit, Catalog No. 74104, and QIAshredder, Catalog No.79654; Valencia, CA) following the manufacturer's protocols. Poly(A) RNA was isolated and used to prepare biotinylated cRNA using the standard Affymetrix (Affymetrix, Santa Clara, CA) protocol (One-Cycle Target Labeling and Control Reagent, Catalog No. 900493). Fifteen micrograms of each labeled cRNA was hybridized to GeneChip Rat Genome 230 2.0 Arrays (Affymetrix) using established laboratory protocols (58). These arrays provide comprehensive coverage of the transcribed rat genome on a single array and are comprised of more than 31,000 probe sets, analyzing over 30,000 transcripts and variants from over 28,000 well-substantiated rat genes.

Data Mining

GeneSpring GX v 7.3.1 (Agilent Technologies, Wilmington, DE) was employed in the mining of the DNA microarray data. Data normalizations were set to fix low abundance genes with relative signals below 0.01 to 0.01. This prevents the software from recognizing large-fold changes in low abundance genes possibly because of background fluctuations. Then differences between chips were normalized with respect to the mean levels of 56 sequences in current use as RT-PCR controls. These included various GAPDH, peptidylprolyl isomerase, and FK 506 binding protein isoforms, but omitted actin-related sequences because cadmium perturbs f-actin assembly (44, 45, 59), and potentially could alter actin mRNA levels by some feedback mechanism. After chip normalization, the mean expression level of each gene in the cadmium-exposed rats was normalized relative to the mean expression of the same gene in age-matched control rats. Other filter criteria for identification/confirmation of discriminatory genes included: its expression level must be >200 in at least one sample (control or cadmium-exposed), and the sort score must be >2.0 (similar to a measure of relative confidence) in the GeneSpring GX algorithm. Genes that met these criteria were further studied.

For statistical comparisons, we employed the SigmaStat v 3.0 software package, and we compared normalized gene

expression levels in control animals with expression levels in exposed animals by Mann-Whitney rank expression, reporting these P values. To obtain estimates of standard errors of the mean, we applied a t test to the same data, even if the data set failed the Kolmogorov-Smirnov normality test with the threshold $P = .20$. Data is presented as mean levels with standard error of the mean.

RESULTS

Chronic Low-Dose Cadmium Exposures Result in a Time- and Dose-Dependent Reduction in Sperm Motility

No sperm were found in the epididymes of animals exposed to cadmium for 1 week, as they were just beginning puberty. At 4 weeks of cadmium exposure, cadmium produced a dose-dependent inhibition of epididymal sperm motility (analysis of variance [ANOVA], $P < .007$). After 8 weeks of cadmium exposure, sperm motilities were lower than at 4 weeks at each cadmium dose employed (t test, $P < .025$ – $P < .001$), and could not be differentiated statistically by dose (ANOVA, $P = .239$, NS). In all cases, sperm motility was $<10\%$ of control after 8 weeks of cadmium treatment.

Effect of Cadmium on Expression of Genes Modulated by Calcium

We asked if a set of 20 genes whose expression is modulated directly or indirectly by calcium was perturbed by cadmium exposure. Figure 1 shows results for a few of these genes. Expression levels of genes with serum response element (SRE)-like enhancer sequences (e.g., nuclear receptor subfamily 4, group A, member 2; Nr4a2) as well as genes containing other calcium-responsive elements (neural cell adhesion molecule 1' [Ncam1'] and a gene similar to Nr4a2 downstream protein 2 [MGC105647]) were perturbed by cadmium exposure.

Genes Coding for Calcium Binding Proteins

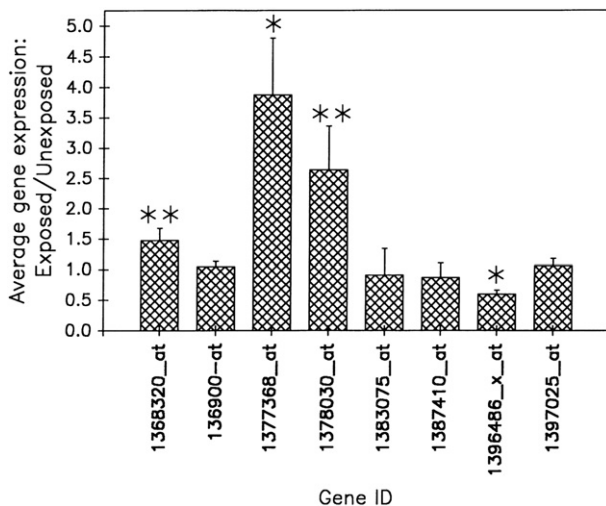
We compared expression levels of calmodulin 1 (Gene IDs: 1387772_at, 1369936_at, 1369937_at), calmodulin 2 (Gene ID: 1370246_at), calmodulin 3 (Gene IDs: 1370837_at, 1368101_at), a predicted calmodulin-like 3 gene (Gene ID: 1384836_at) and CaMK IV (Gene IDs: 1391142_at, 1369752_a_at, 1369753_at, 1375598_at) levels in rat testes exposed to 100 mg/L cadmium for 8 weeks versus 8 week no cadmium controls. Except for two CaMK IV variants, all were decreased 10%–20%, much less than genes modulated by calcium in Figure 1. None of these decreases were statistically significant (Mann-Whitney tests, $P = .1$ to 1.0, not significant).

Splicing of Calcium Channel mRNAs

Figure 1 shows genes coding for different isoforms of the same protein were differentially affected by cadmium (e.g., the two Ncam1 mRNAs). Therefore, to examine the effect of cadmium on protein isoforms documented to be produced

FIGURE 1

Genes from calcium/cAMP signaling pathways respond to chronic cadmium exposures. The genes are denoted by their Affymetrix gene identification numbers. These correspond to, in order left to right, gene names NCam1, Nr4a2, NCam1', Picalm, Ccnd1, Nr4a2', MGC105647, and Trpc4. NCam1 and NCam1' are isoforms of neural cell adhesion molecule 1, Ccnd1 is cyclin D1, Picalm is phosphatidylinositol binding clathrin assembly protein, Nr4a2 and Nr4a2' are isoforms of nuclear receptor subfamily 4, group A, member 2. MGC105647 is similar to Nr4a2 downstream protein 2, and Trpc4 is an isoform of transient receptor potential cation channel, subfamily C, member 4. Each RNA expression level averages microarray results from testes of five Wistar rats. Expression levels of all genes on each microarray were normalized with respect to 56 housekeeping genes to control for chip-to-chip variability. The average level of each gene in the age-matched Wistar (no cadmium exposure) rats was set to 1 to show relative expression levels in the treated (100 mg/L cadmium in drinking water for 8 weeks) Wistar rats. The cadmium dependence of expression of genes with calcium-dependent transcriptional controls and SRE-control elements argues chronic cadmium exposures modulate gene transcription by competing with calcium ion signaling. Expression of NCam1' and MGC105647 (*) in cadmium-exposed testes differed significantly from no cadmium controls (Mann-Whitney tests, respectively, $P < .032$ and $P < .008$). Expression of NCam1 and Picalm (**) displayed a trend that likely would approach statistical significance if sample size were to be increased (Mann-Whitney tests, respectively, $P = .1$ and $P = .07$).



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by alternate splicing of pre-mRNAs (43), we examined the expression of L-type calcium channel subunits.

Figure 2 contrasts effects of cadmium exposure on levels of five different L-type voltage-dependent calcium channel (L-VDCC) alpha-1D pore-forming subunit splice variant sequences, three fully sequenced isoforms, and two ESTs. 1370637_at (6975 bases; from rat brain) expression level increases 50% by chronic cadmium exposure, 1369086_a_at (7986 bases) increases 40%, and 1378950_at (549 bases) increases 17%. Changes in levels of the levels of the other two genes were not significant.

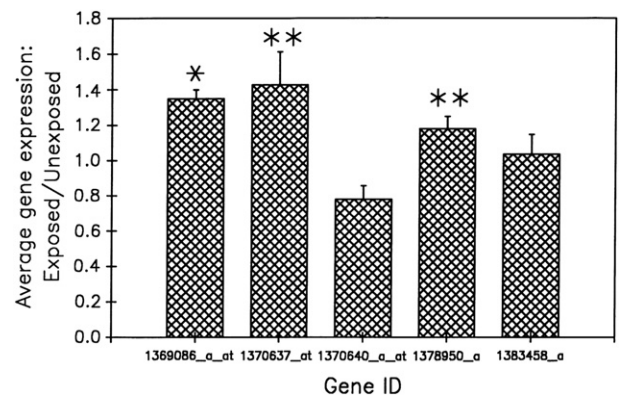
We observed similar cadmium dependence of mRNA expression when we looked at L-VDCC alpha-1C genes, and at accessory beta and gamma subunits (results not shown).

DISCUSSION

A male factor may contribute to the infertility of at least 50% of all couples seeking an infertility evaluation, and asthenozoospermia (low sperm motility) alone may be responsible for the production of this male factor in 19% of all men

FIGURE 2

Effect of cadmium on different L-type voltage-dependent calcium channel (L-VDCC) alpha-1D pore-forming subunit sequences. The same samples are compared as in Figure 1. Data shown is for the 8-week cadmium exposed data set. Similar findings were obtained with the 1-week exposure set. The different L-VDCC alpha-1D splice variants are identified by Affymetrix gene identification numbers. Expression of 1369086_a_at (*) in 8-week cadmium exposed testes was significantly increased compared with no cadmium controls (Mann-Whitney test, $P < .032$). Expression of 1370637_at and 1378950_at (**) displayed a trend that likely would approach statistical significance if sample size were to be increased (Mann-Whitney tests, respectively, $P = .1$ and $P = .09$).



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studied and contributory to another 63% in combination with other semen parameter abnormalities (60). Therefore, studies relating motility and fecundity seem important in several ways. First, the establishment of threshold values for motility and fecundity seem clinically relevant, because these values may dictate when to treat and the approach to therapy. Second, because current treatments for asthenozoospermia are nonspecific or involve costly assisted reproductive technologies (IVF/intracytoplasmic sperm injection) that minimize or bypass the requirement for sperm motility, studies related to sperm motility and fecundity seem important for future clinical advancements.

In one such study, semen parameters and pregnancy outcomes were recorded for couples undergoing intrauterine insemination, and these data resulted in a clinical classification based on threshold values for motility and male fertility. These data suggest that fertile men have >63% motile sperm, subfertile men have <32% motile sperm, and men with indeterminate fertility have >39% but <63% of sperm with motility (61). In separate studies, sperm motility seemed predictive of semen donor fecundability (62, 63). Although these clinical findings may help determine an immediate treatment plan, it seems obvious that a better understanding of the mechanisms involved in aberrant motility is desirable to achieve meaningful advances for these men.

To date, only a limited number of specific disorders have been identified that are available for the study of aberrant motility. These include primary ciliary dyskinesia (Kartagener syndrome), dysplasia of the fibrous sheath of the sperm flagellum, retinitis pigmentosa, and Usher syndrome; for review, see (32). More recently, mutations in two calcium-permeable ion channels, CatSper2 and polycystin-2, have been linked to the production of asthenozoospermia; for review, see (35, 43). Although studies on these conditions have produced interesting genetic and molecular information, their scope is limited. The etiology for the majority of cases with poor motility remains unknown. Based on our prior findings (9, 13, 44–46, 63), we hypothesize that environmental factors contribute to some of these cases.

We tested our hypothesis in a rat model. Although low-level, chronic background cadmium exposures are widespread (9), current understanding of cadmium-related testicular insults in animal models is based on studies of acute effects of a single high-dose cadmium injection, well above typical chronic low-level environmental exposures. Intraperitoneal, subcutaneous, and/or intravenous injection is not the normal way human males are exposed/consume cadmium. Current thinking in the toxicology forum is that experimental animal models should be as physiologically relevant to humans as possible, and that administration of chemicals, such as cadmium, to animals should be via the same route as human exposure. Therefore, we employed chronic, oral, low-dose cadmium exposures.

To the best of our knowledge, only a few studies have addressed the effects of orally administered, environmentally

realistic low-dose cadmium (5 mg/L to 100 mg/L) (52–54) on tissues of the male reproductive tract; see (38, 53, 65), and references therein. The majority of these studies indicate that, when cadmium is administered orally and chronically, the testis accumulated about four times more cadmium than after single-bolus, high-dose cadmium injection. However, changes in testis histology progressed more slowly and were much less dramatic than after parenteral administration. Nonetheless, significant changes in gene expression occurred and were detectable within 72 hours of initiation of cadmium exposure. The latter indicated that changes in gene expression occur well before overt effects of cadmium-induced reproductive toxicity become apparent. Our study differs from those that preceded it, as ours is the first to systematically examine the effects of time and dose in conjunction with low-dose cadmium.

Herein we present the findings from a pilot study. We show that genes whose expression is under calcium control were modulated by cadmium exposure (see Fig. 1). We have not characterized the signaling pathways affected by cadmium as yet, and cannot explain why some genes are enhanced and some suppressed. However, that multiple calcium-dependent response elements were involved agrees with results from cadmium carcinogenesis studies showing that cadmium acts, at least in part, by altering calcium homeostasis (66).

We then asked if levels of genes coding for calcium-binding proteins were affected by cadmium exposures. The genes examined (calmodulin and CaMK IV) were chosen because existing literature suggests they may be involved in sperm flagellar flexure. One hypothesis is that cadmium affects sperm motility by occupying calcium binding sites on calmodulin. This hypothesis implicitly assumes that calmodulin levels are not changed by cadmium exposure. The data we obtained is reasonably consistent with this assumption.

Finally, we examined the relationship between cadmium exposure and expression of different splice variants for a number of genes. Of particular interest was the detection of alternative splicing of L-VDCC genes (e.g., Fig. 2). We suspect that these reflect real differences in protein levels, and correspond to changes in isoform expression. We have previously reported that alternative splicing of the human testicular L-VDCC alpha-1C subunit mRNA, potentially controlled by CaMK IV, is driven by elevated cadmium levels (43, 64). We now report a similar situation occurs in rat testes exposed to cadmium. In addition, for the first time, we report that L-VDCC alpha-D mRNA splicing is also driven by cadmium exposure. Simultaneous expressions of truncated L-VDCC alpha-1D mRNAs and proteins together with full-length products have been reported in human T-cells (67), where they were hypothesized to modulate the channel characteristics. Because L-VDCC alpha-1D activities are modulated by protein kinase A (68) and calmodulin (69), the cadmium exposure may also alter the response of the various L-VDCC alpha-1D isoforms to secondary signals propagated by calcium or its metal ion competitors within the testes. Since L-VDCC

alternatively spliced mRNAs studied encode proteins that localize to the plasma membrane of the sperm tail (43, 70, 71), and since inhibitors of L-VDCC block sperm motility (71–74), our data further support a role for these ion channels in regulation of sperm motility. Thus, it is likely that cadmium affects sperm motility at the level of calcium entry by altering calcium channel properties.

Both influx of extracellular calcium and calmodulin signaling participate in regulation of sperm motility. As [1] L-VDCC mRNA levels, but not calmodulin mRNA levels, are changed by cadmium exposure, [2] L-VDCC are also permeable to cadmium (43, 75, 76), and [3] L-VDCC antagonists such as dihydropyridines block intracellular cadmium accumulation while channel agonists enhance cadmium uptake (76–78), these data suggest that the sperm tail L-VDCC are the primary site of cadmium toxicity in relation to sperm motility. Taken together, these data lead us to hypothesize that sperm tail calcium channels may be developed as pharmacologic targets to improve sperm motility defects results from toxic environmental exposures.

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