

Breakthroughs in Andrology

The Human Acrosome Reaction Is Highly Sensitive to Inhibition by Cyclodiene Insecticides

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ABSTRACT: The mammalian sperm acrosome reaction (AR) is essential to fertilization. It can be initiated *in vitro* by progesterone, a putative physiological initiator that helps to activate sperm GABA_A receptor/chloride channels and by glycine, a substitute for the egg zona pellucida, which activates sperm glycine receptor/chloride channels. Even at 1 nM (0.41 ng/ml or 0.41 ppb), chlordane and endosulfan, chlorinated cyclodiene blockers of insect neuronal GABA_A receptor/chloride channels, strongly inhibited the AR initiated by

progesterone or glycine. Inhibition of the latter was also seen at 0.1 nM chlordane and endosulfan, but neither cyclodiene inhibited either AR initiator at 0.01 nM. Inhibitory concentrations of these cyclodienes are well within the range detected in human and wildlife tissue and fluids as a result of environmental contamination.

Key words: Sperm, fertilization, chlordane, endosulfan.

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Sperm amino acid neurotransmitter receptor/chloride channels are involved in the mammalian sperm acrosome reaction (AR), an essential fertilization event (Meizel, 1997). AR initiation *in vitro* by the egg zona pellucida and by progesterone, a putative physiological AR initiator, involves GABA_A receptor/chloride channels and glycine receptor/chloride channels, respectively (Wistrom and Meizel, 1993; Melendrez and Meizel, 1995; Shi and Roldan, 1995; Melendrez and Meizel, 1996). Chlorinated cyclodienes are a group of potent insecticides that act as blockers of neuronal GABA_A-gated chloride channels (Casida, 1993; Bloomquist, 1996). Their persistence in the environment, along with other organochlorine compounds, has been a matter of concern (Simonich and Hites, 1995). The chlorinated cyclodienes chlordane and endosulfan have been detected in components of the food chain, including dairy products (Maitre et al, 1994; Bentabol and Jodral, 1995) and fish (Kennish and Ruppel, 1997). Endosulfan and chlordane can also block GABA_A receptor/chloride channels in the mammalian nervous system, resulting in convulsions (Pomes et al, 1994; Rosa et al, 1996), and can have damaging effects on other organs,

including the liver (Capen, 1994) and testis (Sinha et al, 1995). The use of chlordane but not endosulfan has been severely restricted in some countries, including the United States. However, both insecticides are still used in a number of countries (including developing nations), and chlordane and endosulfan still persist in the environment in those countries in which it had been used (Maitre et al, 1994; Simonich and Hites, 1995; Kennish and Ruppel, 1997). In view of the importance of amino acid neurotransmitter receptor/chloride channels to the AR and the widespread use or persistence of chlordane and endosulfan, we investigated whether these two cyclodienes would have any effect on the human acrosome reaction initiated *in vitro* by progesterone or by glycine, a replacement for the zona pellucida (Melendrez and Meizel, 1995). The results of these experiments demonstrate that chlordane and endosulfan are highly potent inhibitors of the human sperm acrosome reaction.

Materials and Methods

Materials

We purchased chlordane (isomer mix) and endosulfan (57.3% alpha and 42.7% beta) from Chem Service (West Chester, Pennsylvania); Pentex Fraction V bovine serum albumin (BSA) was purchased from Miles Inc. (Kankakee, Illinois) fluorescein isothiocyanate-labeled Concanavalin A lectin (FITC-Con A lectin) was purchased from EY Laboratories (San Mateo, California).

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Progesterone and glycine were purchased from Sigma Chemical Co. (St. Louis, Missouri); we obtained 15-ml Blue Max conical polypropylene tubes from Fisher Scientific Co. (Fairlawn, New Jersey). All other chemicals were of reagent grade and were purchased from Sigma, Fisher, or Mallinkrodt Inc. (Paris, Kentucky). Deionized water was further purified to $X \geq 18$ megohms-cm using a NANO-pure system (Barnstead/Thermo-dyne, Dubuque, Iowa).

Sperm Capacitation

Sperm require cellular changes *in vitro* or *in vivo*, collectively known as capacitation, before they can respond to AR initiators (Yanagimachi, 1994). In the present study, human semen was obtained from eight healthy donors (medical students, graduate students, and postdoctoral associates) by masturbation. A sperm population having >95% motility was obtained by discontinuous Percoll gradient centrifugation as previously described (Thomas and Meizel, 1988). The gradient-prepared and washed sperm were then diluted to 6×10^6 /ml in a bicarbonate-buffered medium composed of a modified Tyrodes solution containing lactate, pyruvate, glucose, streptomycin, Penicillin G, and 26 mg/ml Pentex Fraction V BSA. Sperm (500- μ l aliquots in 15-ml polypropylene tubes) were capacitated by incubation at 37°C in a humidified 5% CO₂/95% air atmosphere for 24 hours (Turner and Meizel, 1995).

Preincubation with Cyclodienes and Addition of AR Initiators

The 500- μ l aliquots of capacitated sperm were preincubated at 37°C in a humidified 5% CO₂/95% air environment for 5 minutes with various concentrations of chlordane or endosulfan or with cyclodiene solvent (0.001% DMSO in water or, in one study, 0.0005% DMSO in water). Progesterone (3.18 μ M), glycine (225 μ M), or solvent (0.05% DMSO in water for progesterone, and in one study for glycine, or water for glycine) was added to the sperm. Incubation was continued for 2 minutes under the same conditions used for preincubation.

A study was also made of the effect of pretreatment of capacitated sperm with 500 nM chlordane, 500 nM endosulfan, or solvent (0.001% DMSO in water) on the AR initiated by the calcium ionophore ionomycin or its solvent (0.5% ethanol). After preincubation with cyclodienes, sperm were incubated for 2.5 minutes with ionomycin followed by quenching of the ionophore with extra BSA, all as previously described (Thomas and Meizel, 1988) except that 9 μ M ionomycin was used.

In studies with each AR initiator, after motility estimates were made, sperm were fixed and assayed for AR (as described below). Samples were coded and not identified until after AR assays.

Motility Assays

Subjective estimates of the percentage of motile sperm and the quality of sperm motility were made at 500 \times immediately after AR initiation (Thomas and Meizel, 1988; Meizel et al, 1997). Quality was established by a subjective estimate using a scale from 1–4, with 4 indicating that most sperm have strong forward motility and 1 representing twitching and a lack of forward movement (Thomas and Meizel, 1988). In three experiments, the

actual percentages of motile sperm were determined in progesterone-treated sperm that had been pretreated with 1 and 100 nM endosulfan, 1 or 100 nM chlordane, or solvent control (0.001% DMSO in water) for 5 minutes. Two hundred sperm were counted in each sample within 3 minutes after progesterone addition.

AR Assay

Sperm were fixed with 4% formaldehyde for 1 hour prior to staining with FITC-Con A lectin (Meizel and Turner, 1993). FITC-Con A lectin binds to the inner acrosomal membrane of the acrosome-reacted sperm, and those sperm display anterior head fluorescence. The percentage of acrosome-reacted sperm was determined for each treatment by counting the number of AR in 200 sperm, and the percentage data was arcsine transformed. A complete randomized block of the data for daily variation was used to form an analysis of variance table. Significance ($P \leq 0.05$) was determined using the Dunnett one-tail post hoc test.

Results

The results shown in Table 1 demonstrate that preincubation of capacitated sperm with chlordane or endosulfan at concentrations between 500 nM and 1 nM can significantly and strongly inhibit the human AR initiated by progesterone or glycine. Figure 1 shows the effects that preincubation of capacitated sperm with lower concentrations of chlordane or endosulfan (1 nM–0.01 nM) have on the progesterone-initiated human AR. Significant and strong inhibition of the AR is seen again at 1 nM chlordane or endosulfan (75% and 81%, respectively), but significant inhibition does not occur at 0.1 nM or 0.01 nM chlordane or endosulfan. Figure 2 depicts the effects that preincubation with lower concentrations of chlordane or endosulfan (1 nM–0.01 nM) have on the glycine-initiated human AR. In this case, significant and strong inhibition is seen at both 1 nM and 0.1 nM for chlordane (69% and 49%, respectively) and at 1 nM and 0.1 nM for endosulfan (95% and 54%, respectively). No significant inhibition was detected at 0.01 nM for either cyclodiene. All percentage inhibitions were calculated after subtraction of solvent control mean values.

We also tested the effect of preincubation of each cyclodiene (500 nM) on the AR initiated by the calcium ionophore ionomycin and found that neither insecticide inhibited the AR initiated by ionomycin (Table 2).

In all experiments, neither chlordane nor endosulfan had any effect on the percentage or quality of motility at any concentration tested. In subjective motility estimates, the percentage of motile sperm ranged from 70–80%, and the motility quality ranged from 2–3. Importantly, the actual estimated percentage and quality of motility were always the same in control and experimental treatments within any single experiment. Moreover, in one series of

Table 1. Effect of higher concentrations of cyclodienes on the human acrosome reaction†

Treatments	Mean % acrosome reaction ± SEM	% Cyclodiene inhibition
Progesterone (3.18 μM)	37.4 ± 5.2*	
Glycine (225 μM)	29.5 ± 3.5**	
Solvent control	13.6 ± 1.2	
500 nM chlordane + progesterone	19.5 ± 3.5	75
50 nM chlordane + progesterone	17.2 ± 1.1	85
1 nM chlordane + progesterone	21.2 ± 3.0	68
500 nM endosulfan + progesterone	17.5 ± 2.6	84
50 nM endosulfan + progesterone	17.0 ± 0.9	86
1 nM endosulfan + progesterone	22.2 ± 3.2	64
500 nM chlordane + glycine	22.4 ± 2.1	45
50 nM chlordane + glycine	19.6 ± 1.1	62
1 nM chlordane + glycine	21.7 ± 4.6	49
500 nM endosulfan + glycine	19.7 ± 2.9	62
50 nM endosulfan + glycine	19.7 ± 2.2	62
1 nM endosulfan + glycine	23.2 ± 2.9	40

† Capacitated sperm (four separate experiments with different donors) were preincubated for 5 minutes with chlordane, endosulfan (500 nM, 50 nM, and 1 nM), or solvent control (0.0005% DMSO in water). Sperm were then incubated for 2 minutes with 3.18 μM progesterone, 225 μM glycine, or the solvent control (0.05% DMSO in water). Acrosome reactions (AR) were assayed with FITC-Con A lectin. Motility was 70–80% in all experiments and was the same in experimental and control treatments within any one experiment. Each asterisk indicates a value for an AR initiator, progesterone or glycine, that is significantly greater ($P \leq 0.05$) than values for solvent control and for that particular AR initiator plus inhibitor. The percent of inhibition was determined after subtracting solvent control values.

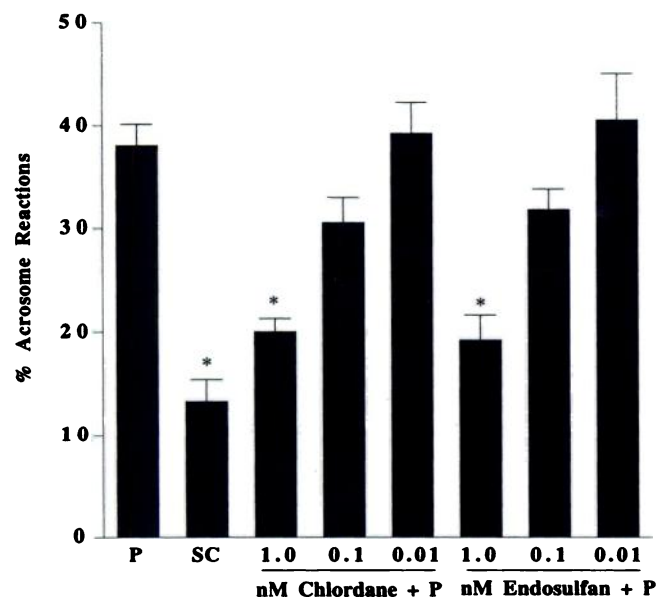


FIG. 1. Effect of lower concentrations of chlordane and endosulfan on the human sperm acrosome reaction (AR) initiated by progesterone (P). Human sperm were capacitated for 24 hours prior to treatment. Capacitated sperm were preincubated for 5 minutes with chlordane, endosulfan (1 nM, 0.1 nM, and 0.01 nM), or solvent control (0.001% DMSO in water) prior to incubation for 2 minutes with 3.18 μM progesterone or its solvent control (0.05% DMSO in water). AR was assayed with FITC-Con A lectin. Error bars represent standard error of the mean for each mean AR value ($n =$ three separate experiments with different donors). An asterisk indicates a value significantly less ($P \leq 0.05$) than that obtained for the addition of progesterone alone. Sperm motility was 70–80% in all experiments and was the same in experimental and control treatments within any one experiment.

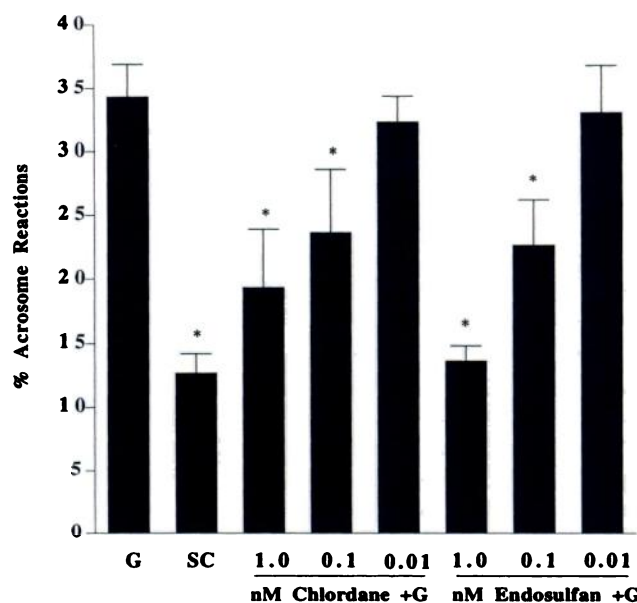


FIG. 2. Effect of lower concentrations of chlordane and endosulfan on the human sperm acrosome reaction (AR) initiated by glycine (G). Human sperm were capacitated for 24 hours prior to treatment. Capacitated sperm were preincubated for 5 minutes with chlordane, endosulfan (1 nM, 0.1 nM, and 0.01 nM), or solvent control (0.001% DMSO) before incubation for 2 minutes with 225 μM glycine or its solvent control (water). AR was assayed with FITC-Con A lectin. Error bars represent standard error of the mean for each mean AR value ($n =$ three separate experiments with different donors). An asterisk indicates a value significantly less ($P \leq 0.05$) than that obtained for the addition of glycine. Sperm motility was 70–80% in all experiments and was the same in experimental and control treatments within any one experiment.

Table 2. Effect of chlordane and endosulfan on the human acrosome reaction initiated by ionomycin†

Treatments	Mean % acrosome reaction ± SEM
Ionomycin	35.0 ± 1.57
Solvent control	10.2 ± 1.73*
Chlordane + ionomycin	36.3 ± 2.96
Endosulfan + ionomycin	34.3 ± 2.19

† Capacitated sperm (three separate experiments with different donors) were preincubated for 5 minutes with chlordane (500 nM), endosulfan (500 nM), or their solvent (0.001% DMSO in water). Sperm were then incubated for 2.5 minutes with ionomycin (9 μM) or its solvent (0.5% ethanol in water). Acrosome reactions (AR) were assayed with FITC-Con A lectin. Motility was 70–80% in all experiments (after quenching ionomycin with additional bovine serum albumin) and was the same in experimental and control treatments within any one experiment. An asterisk indicates AR values significantly different than that of ionomycin ($P \leq 0.05$).

three experiments with different donors, we also counted the number of motile sperm treated with progesterone after pretreatment with 1 nM and 100 nM chlordane, 1 nM and 100 nM endosulfan, or solvent. Motility (mean ± SEM) in these three experiments ranged from 67.7 ± 5.8% to 72.3 ± 4.9%.

Discussion

The most striking aspects of the present results are that 1 nM (0.41 ng/ml or 0.41 ppb) of two chlorinated cyclodienes, chlordane and endosulfan, can strongly inhibit the human sperm AR initiated by progesterone or glycine, and that even 0.1 nM can inhibit AR initiation by glycine. Chlordane and endosulfan are blockers of insect and mammalian GABA_A receptor/chloride channels (Casida, 1993; Pomes et al, 1994), and some other antagonists of that receptor/channel can also inhibit the glycine receptor/chloride channel (reviewed in Wistrom and Meizel, 1993). Therefore, we suggest that in the present study, these cyclodienes are acting, at least in part, through inhibition of sperm neurotransmitter receptor/chloride channels: a GABA_A-like receptor/chloride channel responsive to progesterone or its metabolites and a glycine receptor/chloride channel responsive to zona protein or glycine (reviewed in Meizel, 1997). *In vitro* studies have shown that 100 nM endosulfan partially blocks GABA_A receptor/chloride channels of mammalian cortical neurons, but lower concentrations were not tested (Pomes et al, 1994). Further studies will be needed to rule out other effects of cyclodienes on human sperm, such as the membrane depolarization effects reported for high concentrations of lindane, another type of organochlorine insecticide (Silvestroni et al, 1997). The calcium ionophore ionomycin stimulates the AR by a mechanism that bypasses the chloride channels. At 500 nM, neither endosulfan nor chlor-

dane inhibited the AR initiated by the calcium ionophore ionomycin. Those results suggest that the cyclodienes are not inhibiting by nonspecific membrane damage and are not interfering with the AR assay, and they support the view that these insecticides are antagonists of sperm amino acid neurotransmitter receptor/chloride channels.

Concentrations of chlordane and endosulfan as high as those used in the present work have been found in bovine milk, bovine cheese, and fish (Maitre et al, 1994; Bentabol and Jodral, 1995; Kennish and Ruppel, 1997) and in human milk (Saleh et al, 1996). There have been few studies of the effects of these environmentally persistent insecticides on reproduction. High concentrations of endosulfan have been shown to inhibit spermatogenesis in laboratory rats (Sinha et al, 1995, 1997). While several organochlorine insecticides, including chlordane, have been detected in the ovarian follicular fluid of women undergoing *in vitro* fertilization (e.g., chlordane levels of 0.1–0.3 ppb), their presence did not appear to interfere with the rate of fertilization or time to cleavage (Jarrell et al, 1993). However, those insecticides would presumably have been diluted to much lower concentrations during *in vitro* fertilization procedures. Moreover, the sperm numbers used in *in vitro* fertilization are many times the number of sperm that reach the egg *in vivo* (Yanagimachi, 1994), and since the insecticides do not totally inhibit the AR, there would still be many sperm *in vitro* that might undergo the AR and be capable of fertilization. Inhibition of the AR among the few sperm that reach the site of fertilization *in vivo* could be a serious problem if endosulfan or chlordane bind to sperm before or after they enter the female reproductive tract. Such binding might be relatively tight since we have found, in preliminary experiments, that washing sperm pretreated with 1 nM chlordane (230–300 volumes of wash medium) did not eliminate inhibition of the progesterone-initiated AR.

There is as yet no established link between human or wildlife infertility, the AR inhibitory levels of chlordane or endosulfan presented here, and the levels of those cyclodienes found in the environment. However, our results suggest that the possible effects on animal and human fertility of these chlorinated cyclodienes and of newer GABA_A receptor/chloride channel blocker insecticides (Casida, 1993; Bloomquist, 1996) warrant investigation.

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