A. 研究の目的

クレアチンキナーゼ(CK)はアデノシン3リン酸(ATP)のγ位のリン酸がクレアチンに転移する反応を触媒する酵素である。サブユニットにはM型（筋型）とB型（脳型）の2種があり、二量体であるため、アイソザイム3種（MM, MB, BB）が電気泳動的に分離される。生理的意義は、筋収縮などエネルギーの大量消費時、ホスホクレアチンを消費してATPを供給することであろう。

化学物質の神経毒性を調べる時、CKはバイオマーカーとしてよく使用されている。私達が行った以前の研究では、1-ブロモプロパン(1-BP)暴露によりラットの中枢神経組織中（大脳、小脳、脳幹、脊髄）CKの活性と量が減少したことを明らかにしたが、この中では、1-BPがラットの生殖器に影響を及ぼすことも明らかになっている。今回の研究では生殖器影響のバイオマーカーとしてのCKの利用可能性を検討した。

B. CK量測定方法の検討

9匹のWistar系雄ラットを断頭して精巣、精巣上体、精嚢と前立腺を切り出し，重量を測定した。右精巣上体を使って、精子の運動率と精子数を測定した。残りの右精巣上体頭部、尾部の組織を冷たいNaH_2PO_4 Buffer（10 mM, pH 5.5）15 mlに入れ細切し、氷上に15分置いた後上清を取った。この上清を3000 rpm, 10分間遠心し、ペレットをNaH_2PO_4 Buffer（10 mM, pH 5.5）で1回洗い、pH 6.8 NaH_2PO_4/ Na_2HPO_4 Bufferでもう1回洗い、最後のペレットは0.5 ml pH 6.8 NaH_2PO_4/ Na_2HPO_4 Buffer中で再懸濁し、-80℃で保存した。

A: Western Blot法によるCKアイソザイム量の測定

精巣上体頭部、尾部、精巣、精嚢と前立腺の膜を取り、各組織に5倍のNaH_2PO_4/ Na_2HPO_4 Buffer（pH 6.8）を加え、精子懸濁液に同じ量の0.1%SDSを加え、ホモジナイズした。ホモジナイズ液を800×gで10分間遠心し、上清をSDS-PAGE（12.5% Acrylamides separating ge)lで電気泳動し、ニトロセルロースに移し，市販のCK-B, CK-M, uMt-CK(ubiquitous mitochondrial creatine kinase)とsMt-CK(sarcomeric mitochondrial creatine kinase)抗体を使って，免疫染色した。

結果はFigs 1〜6で示した。CK-B, CK-M, uMt-CKとsMt-CK抗体、精巣上体頭部の精子原液20 μlではバンドは見つからなかった。CK-BとuMt-CK抗体、精巣上体頭部の精子原液30 μlでもバンドは出来なかった。

B: 酵素免疫法(EIA, enzyme immunoassay)によるCK アイソザイム量の測定

測定方法:

a). 測定組織の前処理

冷凍組織を凍ってる状態で潰して(液体窒素で器具を冷やした状態)、組織粉を凍結したままでチューブに分装し、使用部分だけ処理した。残った粉は-80℃で保存した。

凍結組織粉に、4℃の 50 mM Tris-HCl buffer (pH 7.4, 1 mM EDTA を含む)を組織重量の 5 倍 volume 量で加え、超音波破砕装置を用いて、氷浴しながらホモジナイズし、超遠心用チューブに入れ、45000 rpm (125000 ×g), 4℃, 20 分間遠心し、上清を採取した。

b). 蛋白濃度測定 : Bio-Rad Protein Assay

c). EIA 測定

1. 第一反応 :
   標準抗原 : CK-B (rat), CK-M (rat)
2. 第二反応
3. 翌日:反応生成物である 4-MU を蛍光光度計 (起波長 360 nm, 蛍光波長 450 nm) で測定する (unit: ng/10 μ l)。コンピューターによって、定量計算を行う。

この CK の測定系は愛知県心身障害者コロニー発達障害研究所の加藤兼房先生達により開発され、今回神経制御学部の伊東秀記先生の協力で測定した。その結果、精巣上体から分離した精子の CK-B の量は測定できたが、CK-M はほとんど検出されなかった。

以上の検討結果をまとめると、Western Blot 方法では、CK を多量に含む組織のサンプルについては定量できるが、CK 量が少ない精巣上体から分離した精子サンプルの定量は難しい。酵素免疫法は Western Blot 法より感度が高く、精子中の CK-B、および精巣, 精巣上体中の CK-B, CK-M が定量出来ることが明らかになった。

C. CK-B による人間精子の塗抹標本の免疫染色

CK-B は精子の頭部に存在し、精子の成熟と共に段々減少する。動物では未成熟精子の頭部には CK-B が濃く染められると報告されている (Theo wallimann 1986; Gabor Huszar 1994)。私達は人間の精子の塗抹標本で、市販の CK-B 抗体と愛知県心身障害者コロニー発達障害研究所神経制御学部で精製した CK-B 抗体を用いて免疫染色を行った。市販の抗体ではうまく染まらなかったが、発達障害研究所神経制御学部精製 CK-B 抗体により精子の頭部を染めることができた(図)。今回は定性的な評価にとどまっているが、CK-B の着色度で未成熟精子の割合を求め、男性生殖毒性の評価指標として使うことが出来るかもしれない。

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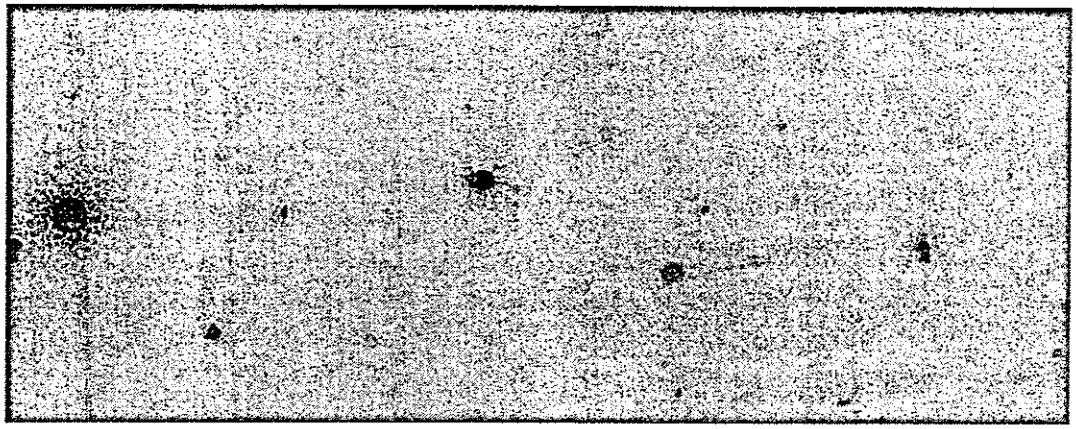
図　精子クレアチンキナーゼ免疫染色



厚生労働科学研究費補助金（化学物質リスク研究事業）
分担研究報告書

化学物質によるヒト生殖・次世代影響の解明と内分泌かく乱作用検出のための新たなバイオマーカーの開発
—　職域集団における男性生殖機能評価研究の実際と課題　—

分担研究者
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要旨

近年、環境化学物質が精液指標など男性生殖機能に与える影響について社会的な関心が高まっているが、一般集団を対象とした疫学研究の実施は、生じうる影響の程度が小さいことに加え、目的とする化学物質以外の交絡要因が多く容易でない。暴露量の多い職域集団を対象とした研究が必要な所以であるが、しかし、国内で実施された研究の数はきわめて少ない。現状を鑑み、職域で精液指標調査を行う際に考慮すべき要因、すなわち、研究デザイン、事業所及び参加者のインフォームドコンセント、被験者の参加率をあげるための工夫、禁欲期間や既往歴など非暴露要因のコントロール、精液指標測定の標準化、結果の解釈、生殖機能が低い結果が得られた場合に配慮すべき事柄について検討した。

III. 研究成果の刊行に関する一覧表

（1）書籍

那須民江（分担執筆） 分子予防環境医学 本の泉社 2003

那須民江（分担執筆） 環境化学物質の代謝とその周辺 日本公衆衛生協会 2003

（2）雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Okamura A, Kamijima M, Shibata E, Ohtani K, Takagi K, Ueyama J, Watanabe Y, Omura M, Wang H, Ichihara G, Kondo T, Nakajima T.	A comprehensive evaluation of the testicular toxicity of dichlorvos in Wistar rats.	Toxicology.	accepted		
上島通浩	職域集団における男性生殖機能評価研究の実際と課題	日本衛生学雑誌	投稿中		
Kishi R, Sata F, Katakura Y, Wang RS, Nakajima T.	Effect of pregnancy, age and sex in the metabolism of styrene in rat liver in relation to the regulation of cytochrome P450 enzymes.	J Occup Health	47	49-55	2005
Wang RS, Suda M, Gao X, Wang B, Nakajima T, Honma T.	Health effects of exposure to ethylene glycol monoethyl ether in female workers.	Ind Health	42	447-451	2004
Kamijima M, Hibi H, Gotoh M, Taki K, Saito I, Wang H, Itohara S, Yamada T, Ichihara G, Shibata E, Nakajima T, Takeuchi Y.	A survey of semen indices in insecticide sprayers.	J Occup Health.	46	109-118	2004
Nakajima T, Yamaonshita O, Kamijima M, Kishi R, Ichihara G.	Generalized skin reactions in relation to trichloroethylene exposure: a review from the	J Occup Health	45	8-14	2003

	viewpoint of drug-metabolizing enzymes.				
上島通浩、柴田英治	臨床医のための「有機溶剤中毒」3．有機溶剤中毒の最近の話題.	日本医事新報	4148	33-36	2003

IV. 研究成果の刊行物・別刷

J Occup Health 2004; 46: 109–118

Journal of Occupational Health

A Survey of Semen Indices in Insecticide Sprayers

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Abstract: A Survey of Semen Indices in Insecticide Sprayers: Michihiro Kamijima, *et al.* Department of Occupational and Environmental Health, Nagoya University Graduate School of Medicine—This study aims at clarifying the semen indices of insecticide sprayers who are exposed mainly to organophosphorus and pyrethroid insecticides. Eighteen male sprayers out of 54 working for 9 companies in central Japan and 18 age-matched students or medical doctors as unexposed controls participated in detailed reproductive check-ups conducted in summer and the following winter. The sprayers were exposed to insecticides more in summer, the busiest season, than winter, the off-season ($p<0.05$). Erythrocyte true cholinesterase activities in the sprayers were lower than in the controls in summer ($p<0.05$), and decreased in significant association with the increase in exposure frequency. Testicular volumes in the sprayers tended to be smaller than in the controls ($p=0.06$). The serum testosterone concentration in winter in the sprayers was higher than in the controls ($p<0.05$), though luteinizing hormone and follicle stimulating hormone concentrations were not significantly different. The sperm counts and vitality were comparable between the groups, but detailed sperm motility analysis in summer revealed that the percentages of slow progressive and nonprogressive motile sperm were twice as high in the sprayers ($p<0.05$), and that of rapid progressive sperm tended to be lower ($p=0.06$). Such differences were not observed in winter. Differential sperm morphology counts showed that interaction of group and abstinence effects were significant in sperm with normal morphology and with head deformity only in the summer check-up. Despite possible inherent differences between the groups, the above season-dependent differences suggested that the observed lower semen quality in the sprayers was associated with pesticide spraying work.
(J Occup Health 2004; 46: 109–118)

Key words: Indoor pesticide sprayer, Organophosphorus insecticide, Pyrethroid insecticide, Occupational exposure, Semen study, Sperm motility, Sperm morphology

The increasing knowledge of the reproductive toxicity of environmental chemicals has raised public concern as to whether the current use of pesticides could adversely affect human reproduction. Among pesticides and their related chemicals, organochlorine insecticides have so far drawn the primary attention. Dichlorodiphenyltrichloroethane (DDT), dieldrin, and toxaphene have intrinsic estrogenic activity and are known as possibly endocrine-disrupting chemicals[1–3]. Lindane and endosulfan decrease testis weight and sperm production in laboratory animals with little effect as endocrine modulators[3–6]. Chlordecone is also known as a testicular toxin in humans as well as in animals[7]. Brominated pesticides or intermediates, 1,2-dibromo-3-chloropropane (DBCP), ethylene dibromide, and 1- and 2-bromopropane, are another group of chemicals with adverse effects on reproductive function[8–15]. Most of these chemicals have been phased out or are strictly regulated for use in developed countries, while benomyl, carbaryl, carbon disulfide, dinoseb, ethylene oxide, fenchlorphos, molinate, triphenyltin, which were reported to have reproductive toxicity in male animals[7], are still in use today.

Given that these pesticides could pose a possible risk for human reproduction, semen studies in pesticide-

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Table 1. Profiles of indoor pesticide sprayers and controls

	n	Age (median, range)	Duration of pesticide spraying work (years) (median, range)	Married/Unmarried Ratio (% married)	With/Without offspring ratio among married (% fatherhood)
Sprayer population	54	35.8 ± 9.5 (34, 21–59)	6.7 ± 6.4 (5, 0.5–28)	32/20[b] (62%)	26/6 (81%)
Examined sprayers	18	33.8 ± 7.0 (33, 24–52)	5.6 ± 5.8 (4.5, 0.5–25)	12/6 (67%)	8/4 (67%)
Controls	18	34.5 ± 7.5 (34, 25–54)	0 (0, 0–0)	11/7 (61%)	9/2 (82%)

	Cigarette smoking			Alcohol drinking[a]			Abstinence period (days)	
	Smoker	Ex	Never	Daily drinker	Occasional	Never	Summer (median, range)	Winter (median, range)
Sprayer population	–	–	–	–	–	–	–	–
Examined sprayers	10	4	4	9	7	2	4.5 ± 2.0 (4.0, 2–8)	4.2 ± 1.4 (4.5, 3–7)
Controls	11	4	3	8	7	3	8.2 ± 9.0[c] (4.5, 3–30[c])	4.8 ± 2.2 (4.0, 3–10)

Age, duration of pesticide spraying work and abstinence period are expressed as the mean ± SD.
[a]Daily drinker, consumes alcohol 5 or more days a week; Occasional, less than 5 d a week; Never, never drinks alcohol. [b]Two sprayers did not provide marital status information. [c]Two controls had abstinence periods of 30 d.

exposed workers have been conducted worldwide in recent years. Some of them suggested the deterioration of semen quality[16–19], while others did not detect any significant alterations[20, 21]. In the light of ongoing development of this research area, it is now necessary to accumulate more findings in various exposure settings.

In this study, we investigated the semen indices of male indoor pesticide sprayers, whose main target species were cockroaches or termites. The sprayers commonly used fenitrothion, dichlorvos (DDVP), chlorpyrifos, and permethrin[22, 23]. Since they often sprayed the insecticides in narrow spaces such as under floors, they could potentially receive extensive exposure[22–26]. Despite the male reproductive toxicity of DDVP suggested in rodent studies[27–29], no human data on exposed populations have been reported to date. As expected, there was great difficulty in conducting the semen study and genital organ examination of 'healthy' subjects, which we managed to overcome. To our knowledge, this is the very first study in Japan to assess the semen indices of workers routinely exposed to chemicals.

Subjects and Methods

Study design and subjects:

The study population consisted of male indoor pesticide sprayers who worked for 9 companies located in the Chubu area, central Japan. These companies were selected from 55 member companies in the local trade association that allowed their employees to participate. At an annual check-up provided under law by the association in March 2000, the 54 sprayers (almost all the male employees in the companies) were asked to undergo detailed examinations focusing on the reproductive system. Among them, 18 (33%) volunteered to participate. As the unexposed control group, 18 students or medical doctors were recruited on an age-matched basis, and all of them participated in the study. The reproductive check-ups were conducted in summer (June through July 2000, average outdoor temperature 25.3°C), the busiest season for pesticide spraying, and in winter (January through February 2001, average outdoor temperature 4.6°C), the off-season. Fifteen sprayers and 16 controls, and 14 sprayers and 15 controls took part in the summer and winter check-ups, respectively. Table 1 shows the profiles of the sprayers and the controls.

Reproductive health check-ups:

The sprayers and controls were asked to abstain from ejaculation for 3–7 days prior to semen sampling. Both groups answered detailed self-completed questionnaires

just before the check-ups on the past history of their jobs, reproduction, illness, long-term prescriptions and febrile illnesses within the past 3 months, subjective symptoms, years of engaging in pesticide spraying work, pesticide names and frequency of their use, and exposure to heat, radiation and other chemicals. Then, a physical examination was conducted by an andrologist (H.H.) along with semen, blood, and urine sampling. Testicular volume was measured with an orchidmeter at the first examination. Semen indices, i.e. volume, pH, sperm counts, sperm motility and morphology, were measured according to WHO guidelines[30]. Briefly, semen samples were obtained after masturbation in a relaxing temperature-controlled room (25–26°C all the year) in a university hospital. Spillage during the semen collection was recorded. The samples were kept under 37°C until liquefaction and examined soon after. Semen appearance and consistency were evaluated, and pH and volume were measured. Then, assessment of motility was performed at a magnification of 400 × on a Thermo Plate (Tokai Hit Co., Inc.) keeping samples at 37.0°C. The motility was graded 'rapid progressive motility', 'slow or sluggish progressive motility', 'nonprogressive motility', or 'immotility'. Sperm vitality was assessed by eosin staining. Counting the spermatozoa was done at a magnification of 400 × with Makler counting chambers, which were proved to give as reproducible and accurate results as the hemocytometer technique. The semen smears were air-dried, fixed in a mixture of ethanol and ether, and stained with a modified Papanicolaou staining procedure. All slides of the smears were randomly coded before analysis, and sperm morphology was evaluated at a magnification of 600 × with no reference to subject identity. Serum concentrations of luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone were measured by radioimmunoassay. Measurements on complete blood cell counts, routine blood biochemistry and urinalysis were conducted to rule out systemic diseases which could affect reproductive function. Erythrocyte true cholinesterase activity (E-ChE) was measured to assess the exposure to organophosphorus (OP) insecticides by an improved version of Garry and Routh's modification of Ellman's method[31]. This study was conducted according to the Declaration of Helsinki; signed informed consent was obtained from all the

Table 2. Pesticides used by 18 indoor pesticide sprayers during the past 1 yr before reproductive check-ups

Pesticides	Number of exposed workers
Organophosphorus insecticides	
Fenitrothion	14
Dichlorvos (DDVP)	11
Chlorpyrifos, Chlorpyrifos-methyl	7
Diazinon	5
Propetamphos	5
Pyrethroid insecticides	
Permethrin	7
Phenothrin	4
Carbamate insecticides	
Propoxur	4

Of the 24 pesticides the sprayers reported, ones which at least 4 workers handled are listed in the table.

Table 3. Symptoms occurring during pesticide spraying

Symptoms	Summer[a]	Winter[a]
Headache	4	4
Vertigo or dizziness	1	4
Lassitude	7	5
Eye irritation	8	4
Delacrimation and nasal discharge	6	5
Nasal and throat irritation	7	6
Coughing and sneezing	9	7
Felt ill during and/or after spraying	7	8

Symptoms at least 4 workers complained of are listed in the table. Figures except for those on the bottom line are sums of the 'frequent' and 'occasional' symptoms. Occurrence of the symptoms was not significantly different between seasons (Fisher's exact test). [a]N=15 in summer and 14 in winter check-up.

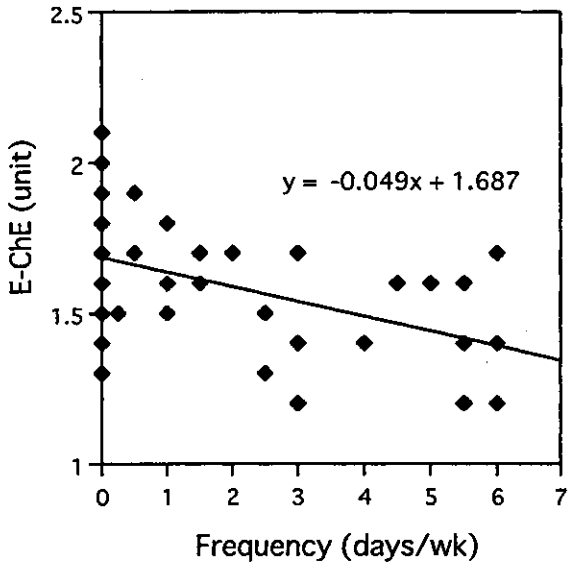

Figure. Relationship between frequencies of pesticide spraying and E-ChE. All data on both sprayers and controls obtained in summer and winter check-ups are plotted in this figure. E-ChE=erythrocyte true cholinesterase activities.

Table 4. Serum sex hormones of indoor pesticide sprayers and controls

Group	LH (mIU/ml)		FSH (mIU/ml)		Testosterone (ng/dl)	
	Summer	Winter	Summer	Winter	Summer	Winter
Sprayer[a]	3.2 ± 1.1	3.7 ± 1.4	6.2 ± 3.2	5.7 ± 3.1	500 ± 133	689 ± 234
Control[b]	2.9 ± 1.5	3.4 ± 1.4	4.9 ± 2.8	5.5 ± 3.0	445 ± 139	470 ± 124
p-value	0.57	0.56	0.22	0.85	0.27	<0.05

The values are expressed as the mean ± SD. LH=luteinizing hormone; FSH=follicle-stimulating hormone
[a]N=15 in summer and 14 in winter check-up. [b]N=16 in summer and 15 in winter check-up.

subjects, and the study protocols were reviewed and approved by the ethical committee of Nagoya University Graduate School of Medicine.

Data analysis:

Frequencies of pesticide exposure were compared by paired *t*-test between summer and winter check-ups. Those of symptoms occurring during spraying were compared between seasons by Fisher's exact probability test. This test was also used to compare the frequencies of varicocele between the sprayers and controls. Blood biochemistry data in each season, including E-ChE, were compared between the two groups with Student's *t*-test. Relationships between exposure frequency and E-ChE and between duration of pesticide spraying work/exposure frequency and sex hormone concentrations were examined with a linear regression model. Since the abstinence periods were not exactly the same for individuals, semen indices in each season were analyzed through 2-way analysis of variance (ANOVA) with interaction to detect the group effect (between sprayers and controls), the abstinence effect (between 'abstinence for less than 5 days' and '5 days or over'), and the interaction of the above two factors. The seasonal factor was not included in the model because the dataset was small and not all the subjects participated in both check-ups. Relationships between duration of pesticide spraying work/exposure frequency and semen indices were analyzed with a linear regression model having the dichotomous variable of the abstinence period. A logarithmic transformation was made for sperm concentration and total sperm counts per ejaculate. A p-value less than 0.05 was considered to indicate a statistically significant difference. JMP ver. 4 (SAS Institute Inc.) was used to analyze the data.

Results

Frequency of pesticide exposure, subjective symptoms during pesticide spraying, and erythrocyte true cholinesterase activity (E-ChE)

The examined sprayers used pesticides listed in Table 2 during the past year. The most frequently used were OP insecticides (mainly fenitrothion, DDVP and chlorpyrifos) followed by pyrethroid insecticides (mainly permethrin). Fenitrothion and DDVP were often sprayed simultaneously as a mixture. The sprayers were exposed to the listed pesticides 3.7 ± 2.2 d per week in summer and 1.3 ± 1.3 d in winter (mean ± SD) with a statistically significant difference between seasons. Nobody in the control group had an occupational history of pesticide exposure.

Symptoms occurring during pesticide spraying are shown in Table 3. They did not differ significantly between seasons. Common problems were 'coughing and sneezing', 'nasal and throat irritation', 'eye irritation', 'lassitude' and 'delacrimation and nasal discharge'. These symptoms often appeared on occasions of ultra-low volume application. Though none of the sprayers had experienced acute intoxication requiring medical treatment, about half of them had felt ill during and/or after the spraying.

E-ChE of the sprayers and the controls were 1.49 ± 0.21 unit and 1.68 ± 0.24 unit with a significant difference in summer and 1.65 ± 0.17 unit and 1.68 ± 0.17 unit in winter (p=0.63), respectively. The figure shows the relationship between E-ChE and pesticide exposure frequency of both sprayers and controls in both summer and winter. E-ChE decreased in significant association with the increase in frequency.

Reproductive check-ups:

Of the 12 married sprayers and 11 controls, 4 sprayers and 2 controls did not have offspring, respectively (Table 1). One in each group stated they were unable to have children even though their spouses were considered to be fertile. The others without offspring had not yet intended to have them. Nobody had a previous exposure history to known testicular hazards. Complete blood cell counts, blood biochemistry (total protein, albumin, total bilirubin, urea nitrogen, creatinine, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, gamma-glutamyltranspeptidase, creatine kinase, serum pseudo-cholinesterase, total cholesterol) and urinalysis (pH, protein, sugar, urobilinogen, bilirubin,

Table 5. Summary of reproductive check-up results of oligozoospermia case (sperm concentration less than 20×10^6/m*l* in summer or winter check-ups)

Case	Age	Group	Duration of pesticide spraying work (yr)	Pesicides used during the past 1 yr	Check-up	Abstinence period (d)	Sperm concentration ($\times 10^6$/m*l*)
A	24	Sprayer	5	DDVP, Fenitrothion, Fenthion, Chlorpyrifos-methyl, Diazinon, Permethrin, Phenothrin, Cycloheximide, Aluminum phosphide	Summer	4	8
					Winter	5	9
B	34	Sprayer	1.5	Fenitrothion, Permethrin	Summer	7	113
					Winter	3	19
C	26	Control	0	None	Summer	3	18
					Winter	3	15
D	28	Control	0	None	Summer	3	35
					Winter	3	13
Reference value							20≥*

	Sperm vitality (%)	Forward progressive sperm (rapidly progressive sperm) (%)	Normally morphological sperm (%)	LH (mIU/m*l*)	FSH (mIU/m*l*)	Testosterone (ng/d*l*)	Varicocele
A	49	44 (25)	1	3.2	4.9	791	None
	59	41 (30)	3	3.3	5.3	830	
B	59	62 (48)	30	5.4	14.9	447	III° (left)
	76	57 (40)	34	5.6	14.3	550	
C	77	64 (52)	52	2.6	6.3	519	None
	90	80 (69)	34	3.5	7.5	627	
D	80	67 (56)	63	1.8	3.0	435	None
	76	51 (43)	54	2.5	4.7	343	
	50≥*	50≥* (25≥)*		1.1–8.8	1.8–13.6	320–1030	

Subjects who showed oligozoospermia in either check-up are listed. *WHO (1999)

ketone body, occult blood) showed no abnormal findings (data not shown). Testicular volumes (mean ± SD) in the sprayers and the controls were 18.4 ± 4.3 m*l* and 21.1 ± 3.8 m*l* for the left (p=0.06), and 19.3 ± 4.0 m*l* and 21.7 ± 3.2 m*l* for the right testis (p=0.06), respectively. Left unilateral varicocele (III° or under) was detected in five participants in the sprayers and three in the controls without a significant difference. Although serum concentrations of LH and FSH as well as other biochemical indices were not significantly different between the groups, testosterone concentrations in winter in the sprayers were significantly higher than in the controls (Table 4). No significant association was observed between the duration of the pesticide spraying work/exposure frequency and these hormonal concentrations (data not shown).

Semen indices:

Individually, two oligozoospermia cases (sperm concentration less than 20×10^6/m*l*[30] in either check-up) were found in each group at least in one of the tested seasons (Table 5). The semen quality of one case in the sprayer group (Case A in Table 5) was characterized by few morphologically normal sperm, lower sperm vitality and lower motility, which was different from all the other subjects in the present study. This subject had been exposed mainly to OP and pyrethroid insecticides for 5 yr. His spraying frequency was lower at the time of check-ups than before. He had felt ill a few times during and/or after spraying, but did not complain of any spraying-related acute symptoms recently. His ChE showed 1.8 unit in summer and 1.9 unit in winter check-ups.

Next, the group effect (sprayer or control) and

Table 6. Semen volume and sperm counts of indoor pesticide sprayers and controls

Group	Semen volume (ml)		Sperm concentration ($\times 10^6$/ml)		Total sperm number ($\times 10^6$)		Sperm vitality (%)	
	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
Sprayer[a]	3.7 ± 1.6	3.5 ± 1.7	72.2 ± 33.6	65.3 ± 37.6	264 ± 128	219 ± 167	67.9 ± 9.2	74.4 ± 8.4
(Median, range)	(3.6, 1.0–6.0)	(3.4, 1.1–6.2)	(72, 8–120)	(65, 9–129)	(286, 38–440)	(196, 36–557)	(67, 49–84)	(76, 56–83)
Control[b]	2.7 ± 1.3	2.8 ± 1.3	94.0 ± 59.6	75.9 ± 57.3	245 ± 235	221 ± 207	69.1 ± 9.9	73.6 ± 8.7
(Median, range)	(2.6, 0.8–4.8)	(2.8, 0.8–5.2)	(77, 18–218)	(65, 13–187)	(204, 39–905)	(182, 61–823)	(69, 52–90)	(73, 49–90)
p-value	0.45	0.25	0.33	0.87	0.19	0.88	0.20	0.96

Values are expressed as the Mean ± SD. *p*-value indicates probability that none of the effects (group, abstinence period, and each interaction) explains a significant proportion of the total variation.
[a]N=15 in summer and 14 in winter check-up. [b]N=16 in summer and 15 in winter check-up.

Table 7. Sperm motility of indoor pesticide sprayers and controls

Group	Motile sperm ($\alpha+\beta+\gamma$) (%)		Rapid progression (α) (%)		Slow progression (β) (%)	
	Summer	Winter	Summer	Winter	Summer	Winter
Sprayer[a]	70.7± 6.2	64.4± 6.4	49.3 ±10.8	49.6 ± 9.4	15.6± 7.1	11.8 ± 4.7
Control[b]	69.4± 4.6	66.7± 6.9	58.1 ± 5.0	53.1 ± 7.2	8.8± 2.8	11.9 ± 3.8
p-value	0.44	0.70	0.06	0.52	<0.05	<0.05
Significant parameter	–	–	(Group)	–	Group	Group Group*Abs

Group	Nonprogressive motility (γ) (%)		Immotile (%)	
	Summer	Winter	Summer	Winter
Sprayer[a]	5.9 ± 2.0	2.9 ± 1.3	29.5± 6.4	35.7 ± 6.9
Control[b]	2.5 ± 1.7	1.7 ± 1.0	30.8± 4.7	33.8 ± 6.9
p-value	<0.05	0.09	0.50	0.79
Significant parameter	Group Abs	–	–	–

Values are expressed as the mean ± SD. *p*-value indicates probability that none of the effects (group, abstinence period, and each interaction) explains a significant proportion of the total variation. Parameter is in parentheses when *p*-value is 0.05–0.10. Abs=abstinence period; *=interaction. [a]N=15 in summer and 14 in winter check-up. [b]N=16 in summer and 15 in winter check-up.

abstinence effect (abstinence for 'less than 5 days' or '5 days or over') were examined for each semen index in the participants in each check-up (Tables 6–8). No statistically significant group effect (sprayer or control) was observed in terms of semen volume, sperm concentration, total sperm number, or sperm vitality (Table 6). Detailed sperm motility analysis showed significant group effects in motile sperm indices in summer (Table 7). The percentages of slow progressive and nonprogressive motile sperm were twice as high in sprayers (15.6 compared to 8.8 in slow progression and 5.9 compared to 2.5 in nonprogression), and that of rapid progressive sperm was lower in the sprayers with borderline significance (p=0.06). By contrast, such differences were not observed in winter (Table 7). Differential sperm morphology counts showed that the interaction of group and abstinence effects was significant in sperm with normal morphology and head deformity only in the summer check-up (Table 8). Finally, analysis of possible relationships between semen indices and duration of pesticide spraying work/exposure frequency revealed no significant association (data not shown).

Discussion

The present study showed possible deterioration in sperm motility of the indoor pesticide sprayers, after adjustment for the abstinence period, compared to the controls in summer, the busy season for sprayers when

Table 8. Sperm morphology of indoor pesticide sprayers and controls

Group	Normal (%)		Head deformity (%)		Middle part and neck defect (%)	
	Summer	Winter	Summer	Winter	Summer	Winter
Sprayer[a]	40.3±13.6	36.3 ± 16.7	54.7 ± 14.6	49.4 ± 15.2	22.5 ± 16.1	34.7 ± 19.3
Control[b]	51.8±10.5	43.9 ± 14.9	41.1 ± 11.1	42.9 ± 12.8	16.9 ± 6.2	25.9 ± 15.6
p-value	<0.05	0.64	<0.05	0.43	0.54	0.56
Significant parameter	Group Group *Abs	–	Group Group*Abs	–	–	–

Group	Tail defect (%)		Cytoplasmic droplets (%)	
	Summer	Winter	Summer	Winter
Sprayer[a]	1.4 ± 1.3	0.6 ± 0.8	0.0 ± 0.0	0.5 ± 0.9
Control[b]	2.6 ± 2.6	1.2 ± 0.9	0.3 ± 0.4	0.8 ± 2.1
p-value	0.32	0.27	0.25	0.32
Significant parameter	–	–	–	–

Values are expressed as the mean ± SD. *p*-value indicates probability that none of the effects (group, abstinence period, and each interaction) explains a significant proportion of the total variation. Abs=abstinence period; *=interaction, [a]N=15 in summer and 14 in winter check-up. [b]N=16 in summer and 15 in winter check-up.

E-ChE was significantly lower than the controls. Sperm morphology analysis revealed significant interaction of group and abstinence effects in the summer check-up, but not in winter, suggesting the existence of summer-only (spraying work-related) change in the sperm morphological dynamism over the sexual abstinence, the sperm transit in the rete testis and epididymis. Thus, these motility and morphology results were in accord with the view that the semen quality of the examined sprayers was lower than that of the control populations in summer. The smaller testicular volume in the sprayer group, though the difference compared to the control group was not significant on each side (p=0.06, requiring an additional 30 subjects in each group to detect such a difference with statistical power (1-β) of 0.9), may also have reflected lower testicular function. We hypothesize that the above results may be associated with the pesticide spraying work. In summer, the higher the frequency of pesticide spraying, the more the sprayers are exposed not only to pesticides but also testicular hazard(s) accompanying the spraying work. One example is a higher scrotum temperature, possibly causing heat stress on the testes[32-34], due to the thick protective pants worn during the high temperature. The sprayers are educated to wear such clothes to reduce dermal exposure to pesticides.

The possibly lower semen quality in the sprayers in this study, who were extensively exposed to OP insecticides along with pyrethroid and other insecticides in summer, is consistent with the findings of other human semen studies conducted in populations occupationally exposed to OP insecticides[16, 17, 19]. Padungtod *et al.* reported that exposure to methyl parathion, ethyl parathion and methamidophos in Chinese pesticide factory workers had a small effect on male reproductive hormones[17] and increased the prevalence of sperm aneuploidy[16]. Another study revealed a positive association between OP metabolite levels and sex null and total aneuploidy frequencies in Mexican agricultural workers[19]. The sprayers in the present study had been exposed to fenitrothion most frequently, the testicular toxicity of which has not been reported, and often sprayed it together with DDVP, which was reported to be a possible testicular toxin[27, 28] and to cause retention of cytoplasmic droplet and reduce sperm motility[29] in animal studies. But it is difficult to attribute the lower semen quality of the sprayer group to specific pesticides at this time. Likewise it remains unknown whether the low semen quality of Case A in Table 5 resulted from insecticide exposure, since no data were available on his semen quality before starting the job.

The present study could not establish a clear dose-response relationship between pesticide exposure and semen indices/serum sex hormones probably due to the small sample size, low exposure level of the examined sprayers, and limited information on exposure to each insecticide. Under the current study design, since the duration from the last spraying of OP insecticides to blood sampling varied greatly depending on the subjects, we did not use E-ChE, an exposure marker for solely OP insecticides (and possibly carbamates), as a variable in

the models to analyze the association of the exposure with the outcomes. In future study, more comprehensive exposure assessment must be considered, especially regarding markers for other than OP insecticides.

Our group previously measured exposure concentrations of permethrin, chlorpyrifos methyl, fenitrothion, and DDVP in a pest control company, one of the member companies of this local trade association[35]. In the measurement, the workers first sprayed 6% permethrin by ultra-low volume application and subsequently 2% DDVP-5% chlorpyrifos methyl or 2% DDVP-5% fenitrothion by hand spray at each work site, still a typical method used by the subjects of the present study. The results showed that the concentration of permethrin was about 10 times higher (max 4.0 mg/m^3) than that of OP insecticides due to the difference in the spraying method, and that of DDVP was about 3 times higher (max 0.36 mg/m^3) than that of chlorpyrifos methyl and fenitrothion due to the difference in their volatility[35]. The above data suggested that the sprayers were exposed to higher concentrations of permethrin and DDVP than other insecticides.

The observed differences in semen quality between the groups as a whole were not striking compared to those found in the DBCP study[8]. Such subtle differences can be attributable to some undetermined factors other than occupational exposure to pesticides. Thus, caution is necessary in the interpretation of the results obtained from this small study. First of all, occupations of the sprayers and the controls were different. Household income, education level, sexual behavior, or unknown factors may be inherently different between groups. Such differences may cause a difference in semen indices between the groups, aside from their levels of exposure to pesticides. Second, the low participation (33%) of the sprayers group could generate a self-selection bias. Although no significant differences were found between the participants and non-participants in terms of age, duration of pesticide spraying work, marital status, and fatherhood in the sprayer population, those concerned about their semen quality for non-occupational as well as occupational reasons could possibly have had a stronger motivation to participate in the study. If the concern arose from non-occupational causes, the deteriorated semen quality could have been attributable to factors other than pesticide spraying. However, the differences in semen quality between groups were more clearly observed in summer when the sprayers were extensively exposed to pesticides. Therefore, the seasonal change in spraying frequency in the sprayers, but not the above possible bias, could better explain the season-dependent differences in semen quality between the groups. In any case, the low participation ratio in the sprayer group was a shortcoming of this study, despite the vigorous efforts to increase participation. This problem is common to semen studies the world over[32, 33, 36].

From other perspectives, three issues remained to be discussed. The first is that testosterone concentrations in the sprayers were significantly higher than in the controls in winter, the off-season for the sprayers. Whether or not this was an exposure-related alteration was a topic of interest and an animal experiment is in progress to answer this question. Second, the semen volume in the sprayer group tended to be higher than in the control group in both summer and winter, although about 50 subjects in each group would be necessary to detect a significant difference with a satisfactory statistical power of 0.9. This was contrary to expectations, and might be related to the higher testosterone concentrations in the sprayers. And, finally, the seasonal variation in the semen quality in the present study showed a reverse trend in both sprayers and controls from other studies in which a decline in the quality, especially in sperm concentration, was observed in summer in North America and Europe[37]. The reason for this discrepancy remains unknown. These issues will be addressed in detail in future studies.

In conclusion, despite the small sample size, low participation and limited information regarding quantification of exposure, it was suggested that the semen quality deteriorated in the indoor pesticide sprayers in summer, the busiest season for pesticide spraying. Nevertheless, caution is necessary in the interpretation of the present results since these changes might have resulted from factors other than pesticide exposure, e.g., higher scrotum temperature under the protective pants worn by sprayers especially in summer. Further studies focusing on seasonal variations in semen indices obtained under strictly-controlled sampling design, time-to-pregnancy, and dose-response relationships on a larger scale, are needed.

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A comprehensive evaluation of the testicular toxicity of dichlorvos in Wistar rats

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Abstract

Assessments of the reproductive toxicity of organophosphorus insecticides are important public health issues. This study aimed at defining the testicular toxicity of dichlorvos (DDVP) since this toxicity was suspected by our previous survey on pesticide sprayers and in some earlier publications during the 1970s.

Ten-week-old Wistar rats were divided into 4 groups (n=8 or 9) and were injected subcutaneously with DDVP (0, 1, 2 or 4 mg/kg) 6 days a week for 9 weeks. After that period, erythrocyte cholinesterase (ChE) activities decreased dose-dependently, showing 44-55% inhibition among the treated groups. No significant difference was observed in the reproductive organ weights in any treated groups compared with the control group. Sperm motility decreased slightly but significantly in the 1 and 4 mg/kg groups, and significant regressions were observed between sperm motility and both blood ChE activity and urinary concentration of dimethyl phosphate (DMP), a urine metabolite of DDVP. However, sperm counts and sperm morphology in the cauda epididymidis, plasma testosterone concentrations, and histopathology in the testes in all the treated groups were not significantly different from those of the control group. Since only the sperm motility deteriorated by DDVP exposure at doses inducing marked inhibition of cholinesterase activities in the rats, it was suggested that the risk of testicular dysfunction posed to occupationally exposed humans would be small in terms of the effect of DDVP exposure alone. This conclusion was also supported by an estimate of the decrease in human sperm motility based on the urinary DMP concentrations observed in actual occupational settings.