Fig. 3 SDS-PAGE, CK-M (N-13)染色

12.5%Acrylamides separating gel

Sample:

1. Testes 5 μl
2. Seminal vesicle 5 μl
3. Prostate 5 μl
4. Epididymides caput 5 μl
5. Epididymides cadua 5 μl
6. Sperms From Epididymides caput 20 μl
7. Sperms From Epididymides cauda 20 μl
8. Testes 5 μl
9. Seminal vesicle 5 μl
10. Prostate 5 μl
11. Epididymides caput 5 μl
12. Epididymides cadua 5 μl

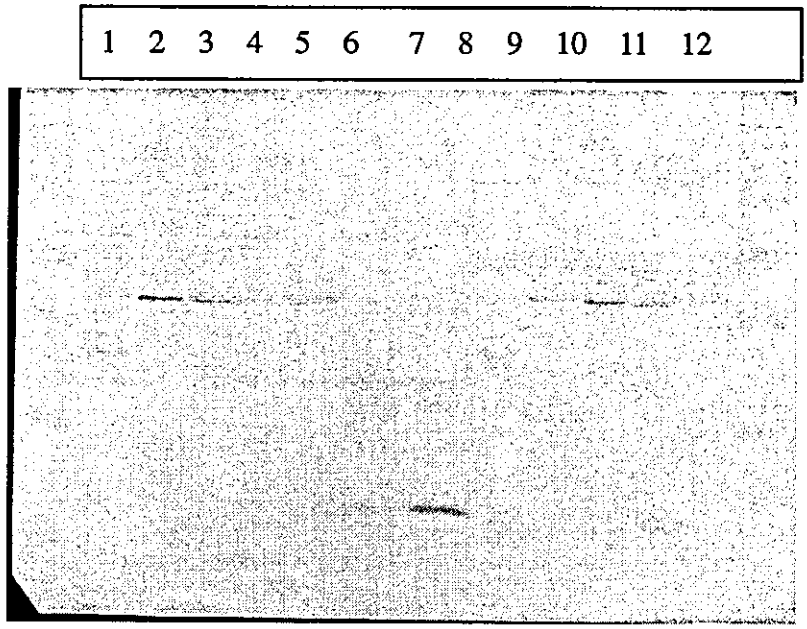


Fig. 4 SDS-PAGE, uMtCK(N-15)染色

12.5%Acrylamides separating gel

Sample:

1. Testes 5 μl
2. Seminal vesicle 5 μl
3. Prostate 5 μl
4. Epididymides caput 5 μl
5. Epididymides cadua 5 μl
6. Sperms From Epididymides caput 20 μl
7. Sperms From Epididymides cauda 20 μl
8. Testes 5 μl
9. Seminal vesicle 5 μl
10. Prostate 5 μl
11. Epididymides caput 5 μl
12. Epididymides cadua 5 μl

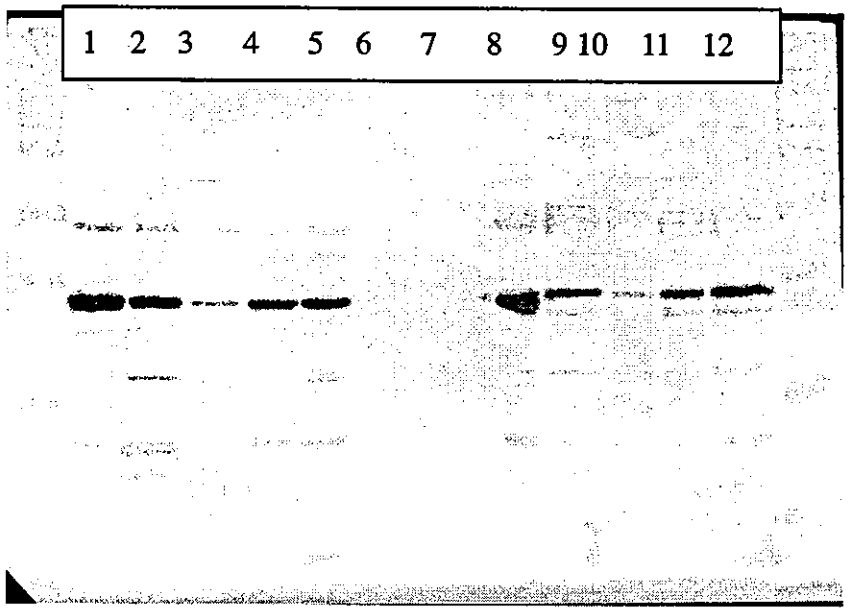


Fig. 5　　SDS-PAGE, uMtCK(N-15)染色

12.5%Acrylamides separating gel

Sample:

1. Sperms From Epididymides caput 30 μl
2. Sperms From Epididymides cauda 30 μl
3. Epididymides caput 5 μl
4. Epididymides cadua 5 μl
5. Testes 5 μl
6. Seminal vesicle 5 μl
7. Prostate 5 μl
8. Sperms From Epididymides caput 20 μl
9. Sperms From Epididymides cauda 20 μl
10. Epididymides caput 5 μl
11. Epididymides cadua 5 μl
12. Seminal vesicle 5 μl

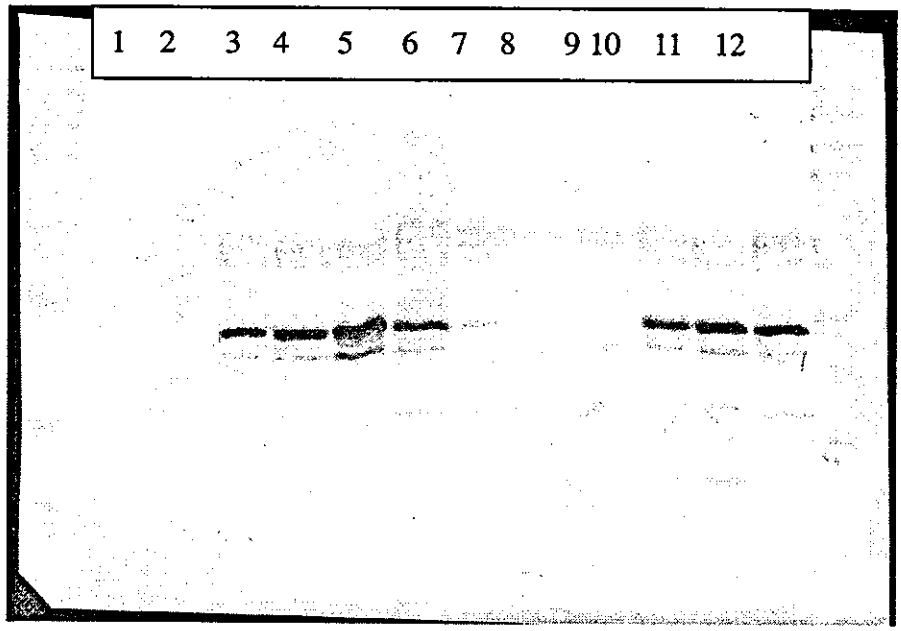


Fig. 6　　SDS-PAGE, sMtCK(C-18)染色

12.5%Acrylamides separating gel

Sample:

1. Testes 5 μl
2. Seminal vesicle 5 μl
3. Prostate 5 μl
4. Epididymides caput 5 μl
5. Epididymides cadua 5 μl
6. Sperms From Epididymides caput 20 μl
7. Sperms From Epididymides cauda 20 μl
8. Testes 5 μl
9. Seminal vesicle 5 μl
10. Prostate 5 μl
11. Epididymides caput 5 μl
12. Epididymides cadua 5 μl

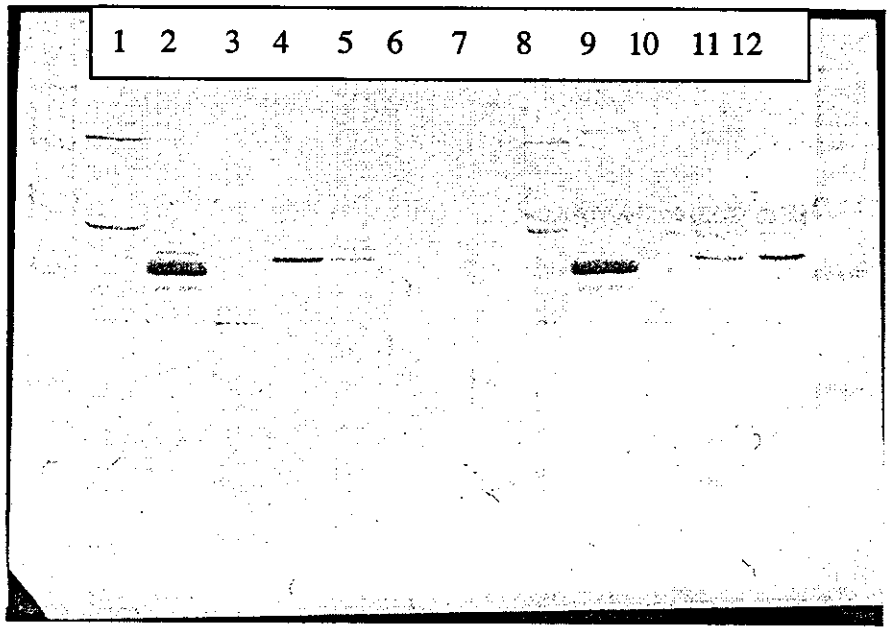
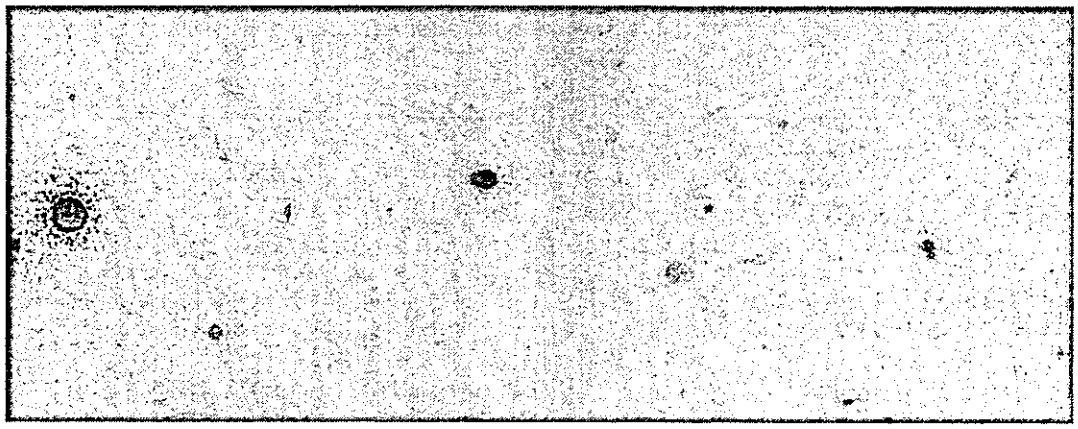


Fig. 7　精子クレアチンキナーゼ免疫染色



III. 研究成果の刊行に関する一覧表

（1）書籍

なし

（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, NakajimaT	A comprehensive evaluation of the testicular toxicity of dichlorvos in Wistar rats	Toxicology	In press		2005
上島通浩	職域集団における精液指標調査研究の実際と課題	日本衛生学雑誌	In press		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

IV. 研究成果の刊行物・別刷

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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 four 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.

Keywords: Dichlorvos; Sperm motility; Cholinesterase activity; Dimethyl phosphate; Organophosphorus; Pesticide; Rat

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1. Introduction

Dichlorvos (2,2-dichlorovinyl phosphate, DDVP) is an organophosphorus insecticide widely used all over the world. Since its commercial introduction in 1961, DDVP has enjoyed extensive use in many countries and has produced important benefits by controlling internal and external parasites in livestock and domestic animals and by controlling insects in houses and fields (ATSDR, 1997). Its annual worldwide sales in 2003 were about 40 million US dollars (Phillips McDougall, 2004). However, the International Agency for Research on Cancer (IARC) regarded DDVP as a possible carcinogen for humans (Group 2B) in 1991, and the US Environmental Protection Agency (EPA) also considered it a probable carcinogen (Group B2) in 1995. Subsequently, in 1995, the US EPA proposed cancellation of DDVP for stored agricultural commodity uses, all home uses, and for many commercial, institutional and industrial area uses; it also proposed strict regulation of retained uses such as application to livestock, animal premises and greenhouses (ATSDR, 1997). In the United Kingdom, the Department for Environment, Food and Rural Affairs (DEFRA) has suspended the sale of a range of products containing DDVP in 2002 because of the carcinogenicity concern (DEFRA, 2002).

From the viewpoint of setting regulations based on the toxicities other than carcinogenicity, the inhibition of acetylcholinesterase is considered to be observable at lower doses than other toxic effects. The reproductive toxicity is not taken into account (ATSDR, 1997; Hummel, 2000). However, in our previous study on the semen indices of indoor pesticide sprayers who often used fenitrothion, DDVP, chlorpyrifos and permethrin, the percentage of slow progressive or non-progressive motile sperm in their busy summer was significantly higher in the sprayers than in the controls (Kamijima et al., 2004). Among the above pesticides, only DDVP was suggested to have any debilitating effects on the male reproductive system in some animal experiments (Krause and Homola, 1974; Krause et al., 1976; Akbarsha et al., 2000), whereas conflicting study results had also been reported (Krause, 1977; NTP, 1989; Dikshith et al., 1976). Since public concern still remains about the possible male reproductive toxicity of DDVP, its comprehensive evaluation is needed, particularly about testicular histopathology and sperm motility.

The present study aims to provide information on the relationship between exposure markers of DDVP and indices of the male reproductive system in the rat under different dose levels. The extrapolation of the present rodent study results to human exposure settings could give a more precise estimate of the risk of testicular toxicity due to DDVP exposure alone, and could provide an answer to the public and occupational health concern.

2. Materials and methods

2.1. Chemicals

DDVP (at least 99.0% pure) was purchased from Kanto Chemical Corporation, Japan. Hanks's solution was obtained from Invitrogen, CA. All staining solutions for testicular histopathology and sperm morphology were purchased from Wako, Japan.

2.2. Animals and treatment

A total of 34 male, 9-week-old Wistar rats were purchased from Shizuoka Laboratory Animal Center, Japan. They were housed and acclimatized to new surroundings for a week under a controlled temperature of 23–25 °C and relative humidity at 57–60%, and then randomly divided into 4 groups ($n = 8$ or 9). Food and water were provided ad libitum.

The rats were subcutaneously injected in the back of the neck with 0, 1, 2, or 4 mg/kg of DDVP which was dissolved in saline. Administration volume was 3 ml/kg. By subcutaneous injection, the absorbed chemical enters the systemic circulation directly, not via the portal system, as in inhalation and dermal exposure. The maximum dose was set to be 4 mg/kg because some rats died during our 9-week pilot study by the daily subcutaneous treatment with 5 mg/kg DDVP, which was beforehand considered tolerable based on the acute oral LD50 (80 mg/kg) (Hummel, 2000) and on the oral dose (10 mg/kg) tolerable for 48 days (Akbarsha et al., 2000). One more rat was assigned for each of the 2 and 4 mg/kg groups in case animals died in the higher-dosed groups. The above treatments were performed 6 days a week for 9 weeks to cover the entire process of spermatogenesis. On the following day after the last injection, rats were weighed, and

sacrificed by collecting the blood into heparinised tubes through the abdominal aorta under pentobarbital anesthesia. The epididymis, testis, prostate, seminal vesicle, liver, kidney and adrenal gland were dissected out and weighed. The present study conformed to Japanese law concerning the protection and control of animals and the Guide to Animal Experimentation of Nagoya University School of Medicine.

2.3. Sperm motility analysis

Sperm were collected as quickly as possible after a rat was sacrificed. The cauda epididymidis was cut by knife to release sperm in 2 ml of Hanks's solution at 37 °C. Sperm in the cauda epididymidis were manually counted under microscopic observation on a warming plate (Tokai Hit Co., Japan), and sperm motility was categorized into "motile" or "non-motile."

2.4. Sperm morphology analysis

The sperm solution was smeared on glass slides, fixed in methanol and dried. The slides were stained later according to Bryan's method (Bryan, 1970). A total of 200 intact sperm were examined for morphological abnormality under the microscope. Abnormal heads were classified as straight, banana-shaped, and other unclassified abnormalities according to the method of Mori et al. (1991).

2.5. Epididymal sperm count

Residual cauda epididymidis were minced with scissors and filtered through gauze. Filtered samples were diluted with saline containing 0.5% formalin. This solution was infused into a Neubauer-type hemocytometer (Erma, Japan) for microscopic observation. The data were expressed as the total number of sperm per cauda epididymal tissue weight. Every sample was evaluated without information about its treatment.

2.6. Histopathological examinations

The right testes were fixed in Bouin's solution and embedded in glycol methacrylate (GMA) (Technovit 7100, Heraeus Kulzer Co., Germany). Tissue sections of the testis were stained with periodic acid Schiff's reagent (PAS) and hematoxylin. The histopathological examination was carried out to evaluate possible changes in the seminiferous tubule level (e.g. atrophy), seminiferous epithelium level (e.g. disorganization, depletion), germ cell level (e.g. degeneration, retention, vacuolation), and Leydig cell level (e.g. degeneration, vacuolation). Three categories of stages (stages I–VI, stages VII–VIII, stages IX–XIV) were all evaluated in every rat without information about its treatment.

2.7. Round spermatid cell counts

Spermatogenic cells at stage VII were counted to evaluate the cellularity of the seminiferous epithelium. Three sections of stage VII in each rat were randomly photographed (Fujix Digital Camera HC-300Z/CL, Olympus Japan Co.). Only round spermatids and Sertoli cells were counted, since alterations in spermatogonia and spermatocytes also result in change in the round spermatids after treatment for 9 weeks.

2.8. Cholinesterase (ChE) activity

Blood samples were immediately centrifuged after their collection and maintained at −4 °C. Plasma and red cell ChE activities were then measured with the modified acetylthiocholine-DTNB procedure (Voss and Sachsse, 1970) within the same day.

2.9. Testosterone assay

Plasma testosterone levels were measured with radioimmunoassay (RIA) by Mitsubishi Chemical BCL Co., Japan. Plasma samples were stored at −80 °C until the analyses.

2.10. Urinary dimethyl phosphate (DMP) concentration

Urinary DMP concentrations were measured by gas chromatography/mass spectrometry equipped with electron ionization (GC/MS-EI), Perkin-Elmer Turbo-Mass Systems (Wellesley, MA) with the modification of a previously-described method (Hardt and Angerer. 2000). Standard curves for measuring DMP in the urine proved to be linear for concentrations ranging from 0.01 to 50 mg/l with a correlation coefficient of 0.999. The intra- and inter-assay coefficients of variation for the GC/MS assay were less than 15%

at concentrations of 0.05 mg/l. The detection limit of DMP was 0.3 μg/l.

2.11. Statistical analysis

Dunnett's multiple comparisons were made between the exposure groups and the control following one-way analysis of variance (ANOVA), when the values were considered to be distributed normally. For the DMP concentration, the Kruskal–Wallis test was used to detect differences between the groups, and Steel's test was used for multiple comparison of the treated groups and the control group. Linear regression analysis was performed for sperm motility versus blood ChE activities or urinary DMP concentration. The DMP value was transformed by root conversion because the standard variation increased in proportion to the mean value. A p-value less than 0.05 was considered statistically significant. JMP ver. 4 (SAS Institute Inc.) was used to analyze the data.

3. Results

3.1. Animals

After about 4 weeks' administration, all rats among the 4 mg/kg group developed severe acute signs of intoxication following injection: tremor, increased salivation, cramps, paralysis of limbs, and reddish secretion from ocular and nasal mucosa. However, the severity of the symptoms differed between the rats and between the days. Some rats administered 2 mg/kg developed much milder acute signs than those of the 4 mg/kg group with inter-individual and inter-day differences. In contrast, the 0 and 1 mg/kg groups did not have any acute signs. The injected sites of the skin were not ulcerated on either rat.

3.2. Reproductive and other organ weight

The body weight at the end of the treatment decreased significantly only in the 4 mg/kg group (Table 1). The absolute weight of testes and epididymides did not change, but their relative weights increased significantly only at 4 mg/kg (Table 1). The absolute and relative weights of the prostate and the seminal vesicle did not change. As for the non-reproductive organs, the absolute weight of the liver decreased with statistical significance, and that of the left adrenal gland significantly increased in the 4 mg/kg group (Table 2). The other organ weights did not show any significant changes.

3.3. Histopathological findings

Histopathologically, a difference in the frequency of abnormality of the testis in all three categorized stages did not differ between the control and any treated groups, while two previous studies showed such

Table 1
Body and reproductive organ weights in rats exposed to DDVP

	0 mg/kg	1 mg/kg	2 mg/kg	4 mg/kg
Number of rats	8	8	9	9
Body weight (g)	426 ± 24	423 ± 14	413 ± 8.5	369 ± 8.5*
Absolute organ weight (g)				
Epididymides (left)	0.58 ± 0.02	0.56 ± 0.07	0.57 ± 0.03	0.57 ± 0.05
Testes (left)	1.62 ± 0.07	1.63 ± 0.11	1.64 ± 0.15	1.64 ± 0.17
Prostate	0.70 ± 0.09	0.80 ± 0.13	0.63 ± 0.24	0.58 ± 0.15
Seminal vesicle	1.96 ± 0.19	2.17 ± 0.21	2.19 ± 0.31	1.88 ± 0.16
Relative organ weight (g/kg body weight)				
Epididymides (left)	1.37 ± 0.11	1.33 ± 0.10	1.39 ± 0.11	1.54 ± 0.16*
Testes (left)	3.81 ± 0.26	3.85 ± 0.22	4.01 ± 0.34	4.45 ± 0.42*
Prostate	1.71 ± 0.21	1.83 ± 0.41	1.81 ± 0.30	1.50 ± 0.38
Seminal vesicle	4.77 ± 0.48	4.98 ± 0.53	5.35 ± 0.68	5.07 ± 0.31

Values are mean ± S.D.
* Significant difference at $p < 0.05$ when compared with control group.

Table 2
Weight of adrenal gland, liver, and kidney in rats exposed to DDVP

	0 mg/kg	1 mg/kg	2 mg/kg	4 mg/kg
Number of rats	8	8	9	9
Absolute organ weight (g)				
Adrenal gland (left)	0.034 ± 0.004	0.036 ± 0.009	0.035 ± 0.012	0.044 ± 0.007*
Liver	12.76 ± 1.73	12.71 ± 0.85	11.69 ± 1.52	10.83 ± 1.89*
Kidney (left)	1.28 ± 0.13	1.32 ± 0.10	1.34 ± 0.04	1.23 ± 0.12
Relative organ weight (g/kg body weight)				
Adrenal gland (left)	0.09 ± 0.02	0.08 ± 0.02	0.09 ± 0.01	0.11 ± 0.03
Liver	29.92 ± 2.84	30.25 ± 1.64	28.36 ± 2.73	29.06 ± 3.42
Kidney (left)	3.06 ± 0.28	3.16 ± 0.17	3.19 ± 0.21	3.25 ± 0.15

Values are mean ± S.D.
* Significant difference at $p < 0.05$ when compared with control group.

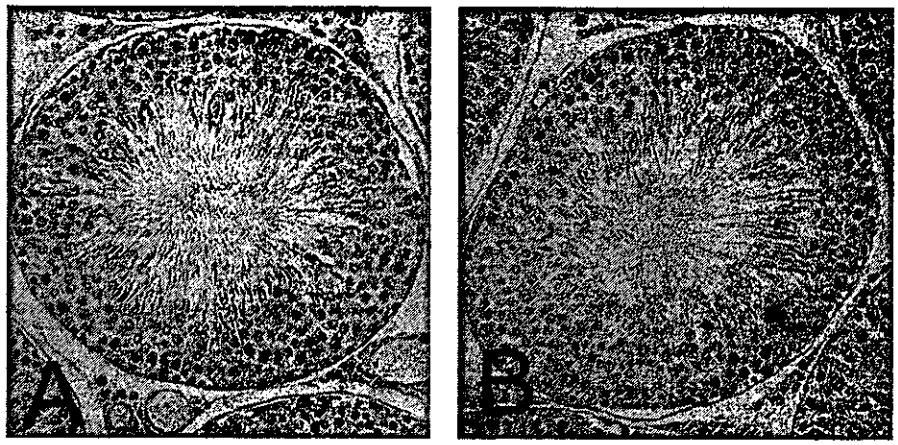

Fig. 1. Stage VII seminiferous tubules of rats subcutaneously injected with DDVP at (A) 0 mg/kg and (B) 4 mg/kg for 9 weeks. No abnormality was found.

changes (Krause and Homola, 1974; Krause et al., 1976). The number of round spermatids per Sertoli cell in Stage VII did not change either (Fig. 1 and Table 3). No vacuolation, exfoliation, degeneration or retention in the seminiferous tubules nor any histopathological changes in intertubular spaces were observed in this study.

3.4. Blood ChE activity and urinary DMP concentration

The ChE activities in erythrocyte and plasma are shown in Table 4. Those in erythrocyte decreased dose-dependently, showing significant 44–55% inhibition in the treated groups. ChE activity in plasma showed

Table 3
Spermatogenic cell count in stage VII seminiferous tubule of rats exposed to DDVP

	0 mg/kg	1 mg/kg	2 mg/kg	4 mg/kg
Number of rats	8	8	9	9
Round spermatids/Sertoli cell	14.2 ± 3.1	15.9 ± 4.7	13.4 ± 1.6	13.5 ± 2.1

Values are mean ± S.D. No significant difference was presented at $p < 0.05$ when compared with control group.

Table 4
Cholinesterase activities in erythrocyte and in plasma

	0 mg/kg	1 mg/kg	2 mg/kg	4 mg/kg
Number of rats	8	8	9	9
Erythrocyte (μmol/ml/min)	1.66 ± 0.20	0.92 ± 0.13*	0.77 ± 0.17*	0.76 ± 0.16*
Plasma (μmol/ml/min)	0.82 ± 0.12	0.74 ± 0.13	0.62 ± 0.11*	0.58 ± 0.10*

Values are mean ± S.D.
* Significant difference at $p < 0.05$ when compared with control group.

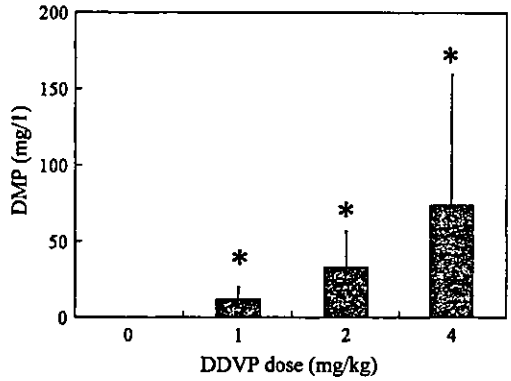

Fig. 2. Urinary dimethyl phosphate (DMP) concentration in rats subcutaneously injected with DDVP. Twenty-four-hour urine was collected after the last DDVP treatment. Each bar represents the mean ± S.D. (*) Significant difference at $p < 0.01$ when compared with the control group.

the same but more moderate tendency with significant decreases only at 2 and 4 mg/kg. DMP in the urine significantly increased dose-dependently with larger standard deviations in higher-dose groups (Fig. 2).

3.5. Sperm motility

Sperm motilities in the cauda epididymidis decreased significantly in the 1 and 4 mg/kg groups but without a clear dose–response relationship (Table 5). The regression of the motility on the urinary DMP concentration and erythrocyte ChE activity were statistically significant (Fig. 3A and B), but not on the plasma ChE (Fig. 3C).

3.6. Sperm morphology

Morphological analysis detected no significant change in sperm head in any treated groups (Table 5). As for morphological abnormalities other than straight and banana-shaped sperm, a twin-tailed sperm was found in only one rat of the 4 mg/kg group.

3.7. Testosterone concentration

Testosterone concentrations in the plasma were not significantly different in any DDVP-treated groups (data not shown).

4. Discussion

This study showed DDVP decreased sperm motility in the rat, whereas no significant changes were found in the histopathology of seminiferous tubules, sperm counts, sperm morphology, histopathology of seminiferous tubules, plasma testosterone concentrations or reproductive organ weights. The extent of the percentage decrease in sperm motility in the treated groups was at most about 14% of the control group.

Table 5
Sperm count, head abnormality and motility

	0 mg/kg	1 mg/kg	2 mg/kg	4 mg/kg
Number of rats	8	8	9	9
Sperm count ($\times 10^6$/g cauda)	314 ± 113	315 ± 93	352 ± 148	349 ± 79
Sperm head abnormality (%)	2.0 ± 1.1	2.9 ± 1.8	3.1 ± 1.4	2.2 ± 1.1
Sperm motility (%)	87.1 ± 2.4	76.8 ± 4.4*	79.2 ± 8.6	74.6 ± 2.2*

Values are mean ± S.D.
* Significant difference at $p < 0.05$ when compared with control group.

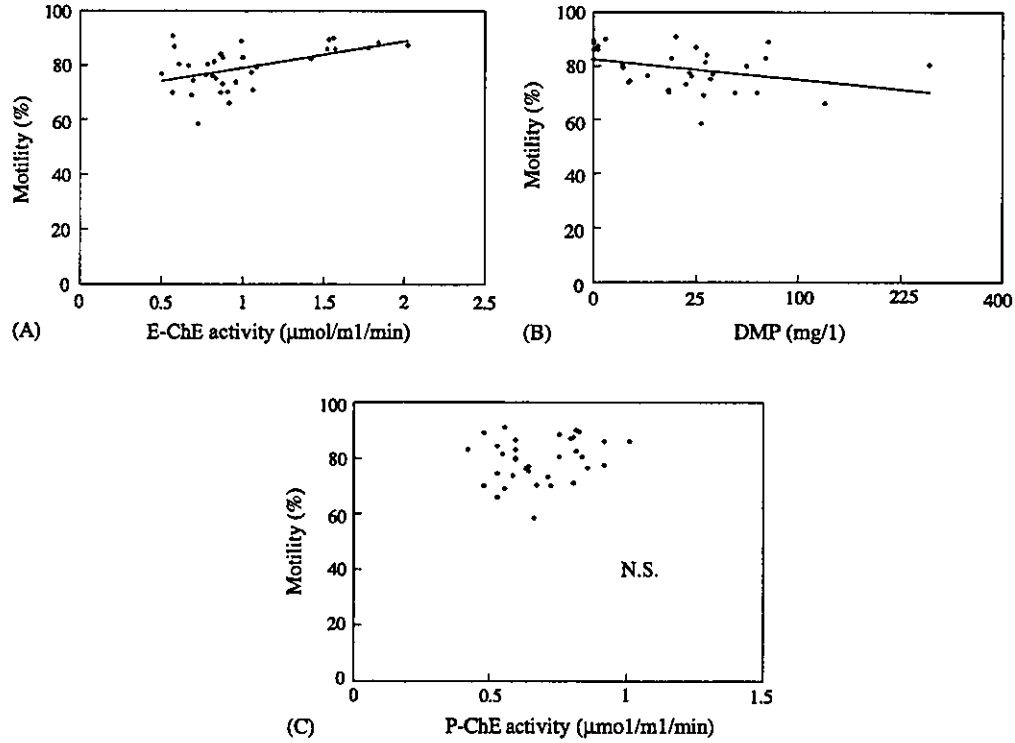

Fig. 3. Regression of sperm motility on internal doses. All data on both exposure and control groups were plotted in the figure ($n = 34$). (A) Regression of percentage sperm motility on erythrocyte cholinesterase (E-ChE) activity was significant ($p = 0.028$). Motility = 9.744 × (E-ChE) + 69.394. $R^2 = 0.2463$. (B) Regression of percentage sperm motility on root-transformed dimethyl phosphate (DMP) concentration was significant ($p = 0.043$). Motility = $-0.7481 \times (\text{DMP})^{1/2} + 82.424$. $R^2 = 0.1215$. (C) Regression of percentage sperm motility on plasma cholinesterase (P-ChE) activity was not significant.

A dose–response relationship was evident between the sperm motility and the internal exposure dose, i.e. blood ChE or urinary DMP. These findings are virtually consistent with a study indicating that the duration time of sperm motility was shorter in rats treated with DDVP (Akbarsha et al., 2000), though the authors did not indicate the percentage sperm motility parameter. They mentioned that the decreased sperm motility might have resulted from the retention of cytoplasmic droplets in the cauda epididymis sperm. Alternatively, mitochondrial enzymatic activity might have been decreased due to DDVP exposure, which in turn decreased sperm motility as reported in an asthenozoospermia patient (Ruiz-Pesini et al., 1998). Sarin and Gill (1999) showed that the activity of cytochrome oxidase (COX) in rat brain exposed to DDVP decreased significantly. Additionally, parathion and methylparathion, other organophosphorus pesticides, inhibited $(Ca^{2+} + Mg^{2+})$-ATPase on the erythrocyte membrane (Blasiak, 1995). Another example is alpha-chlorohydrin (ACH) which inhibits motility of mature sperm in the cauda epididymidis without a decrease in the sperm counts or testicular weights, and without any detectable damage to the epididymis or to the testis under lower doses (Jones, 1983). The mechanism of this inhibition is that ACH inhibits sperm glycolytic enzymes, which results in an inability to maintain continuous ATP synthesis in sperm (Mohri et al., 1975; Brown-Woodman et al., 1978). Thus, the mechanism of decreased sperm motility caused by DDVP exposure needs to be studied further.

When examining at the testis histopathologically in detail, the number of round spermatids per Sertoli cell in stage VII did not change with the treatment, indicating that DDVP did not have an antiandrogenic effect. This result was in accordance with the fact that plasma

testosterone concentrations were not changed. Krause and Homola (1974) observed disturbed spermatogenesis and an increased number and hypertrophy of Leydig cells after DDVP treatment, used too few mice for statistical analysis. Krause et al. (1976) also suggested that spermatogenic cells and Leydig cells were slightly reduced in the testes of juvenile rats exposed to DDVP. However, another study by Krause in 1977 did not reveal any disturbances of the spermatogenetic epithelium. Furthermore, Dikshith et al. (1976) showed that dermal painting of DDVP did not affect the histopathology of skin and testis in rats. In their study, the testes of two rats which had died after five applications of DDVP showed complete necrosis of the majority of the seminiferous tubules, but there was no such testicular damage in the remaining animals with subsequent exposures up to 90 days. In 1989, the National Toxicology Program (NTP) in the US showed no histopathological changes in the testes, epididymes and prostate of rats and mice orally exposed to DDVP for 2 years. Our study results were in accordance with these results that DDVP did not affect testicular histopathology.

As for sperm morphology, the appearance of twin-tailed sperm was reported in DDVP-treated mice (Wyrobek and Bruce, 1975). The report indicated that mouse treated intraperitoneally with 5 mg/kg DDVP for 5 days had about 2.5% abnormality, and even at about 1 mg/kg DDVP induced 1.25% abnormality. However, twin-tailed sperm were virtually absent in the present study. Taken together, although experiments with different animal species/strains, and different routes and doses might result in clearer effects, the results of the previous and our animal studies suggest that the testicular toxicity of DDVP is much milder, and limited to decreased sperm motility or slight increase in morphologically-abnormal sperm than the results of testicular toxic pesticides such as dibromochloropropane (DBCP) (Kluwe et al., 1983).

The final goal of the present study is to estimate the risk of testicular dysfunction due to DDVP in human, though the observed effect in the present study was small and without a clear dose–response relationship. Since the average DMP concentration in the urine of workers occupationally exposed to DDVP was 0.09 mg/l (Saito et al., 1984), the decrease in their sperm motility is anticipated to be 0.22% on a calculation using the obtained regression formula. Even in a person with the maximum DMP excretion of 0.71 mg/l (Saito et al., 1984), the expected decrease in sperm motility would be at most 0.63%. Additionally, erythrocyte ChE inhibition observed in the present rat study was more than 40% in the 1 mg/kg group showing 12% decrease in sperm motility. This does not usually occur even in occupationally-exposed workers. Thus, the risk of testicular dysfunction due to DDVP exposure alone would be small under the usual occupational exposure without a reduction in ChE activities, and would be negligible in general environmental exposure.

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職域集団における精液指標調査研究の実際と課題

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Considerations when conducting a study of semen indices in workplaces

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Abstract

The possible adverse impact of environmental chemical exposure on semen quality is a public health concern. However, an epidemiological study targeting the general population is not easy. An alternative approach is a study of workers exposed to higher doses of the concerned chemicals, while there have been only a very limited number of such studies conducted in Japan to date.

This review, based on our experience so far, attempts to shed light on the considerations when planning possible semen studies in workplaces in Japan. They include study design, informed consent of both the employer and the participating employees, how to increase the participation ratio, control of non-exposure factors such as abstinence period and medical history, standardization in measuring semen indices, interpretation of the study results, and necessary cautions when reporting lower semen quality found in the exposed population.

Key words:職域 workplace、職業性曝露 occupational exposure、精液指標 semen indices、説明と同意 informed consent、疫学研究 epidemiological study

1．はじめに

1992 年、Carlsen らが過去 50 年間でヒト精子数が半減した可能性について報告(1)して以来、環境化学物質が生殖機能に与える影響について関心が高まっている。しかし、一般生活環境中の微量な化学物質の同定・定量が測定機器・技術の進歩により可能になった一方で、これら化学物質への曝露を原因として精子数の減少、精子運動性の低下、形態異常精子の増加などの精液指標の変化がヒトで生じているかについては、いまだ結論が得られていない。これは、曝露が微量な一般環境で疫学研究を実施し、曝露と精液指標との関連を検討することが困難であることを示している。一般生活者集団を対象とした疫学調査が容易でないとすると、これに代わる手段としては、曝露による影響がある場合にそれを観察しやすい集団、すなわち曝露量が多い集団でリスク評価を行い、その結果を用いて曝露量の少ない一般生活者集団のリスク評価を行うことが考えられる。曝露量が多い集団の典型としては、影響の有無を検討すべき化学物質を製造し、または仕事で使用する職域作業者があげられる。このような職域で実施する調査研究は、法定の職域健康管理の枠組みを利用できれば効率よく推進できるはずであるが、残念ながら現状では日本の職域で実施された精液指標に関する研究蓄積はきわめて少ない。その理由は、職域での疫学研究実施に必要な留意事項、精液という一般的でない検体を扱う技術的特殊性に加え、生殖機能という個人のプライバシーに深く踏み込むと多くの人が感じる内容を扱うことのむずかしさにある。フィールド研究として精液指標調査を行う上での留意点に関しては Schrader による総説(2)がほぼ網羅しているが、本稿では筆者のグループが試行錯誤を重ねた経験をふまえ、職域で毒性学的な視点での調査研究を行う場合に最も問題となる、事業所や研究参加者とのコミュニケーションの内容を含め、日本国内の職域で実施する精液指標調査のあり方について明らかにしたい。

2．職域における精液指標調査研究の疫学

生体試料としての精液の入手は尿や血液等に比べ困難であることから、精液採取を伴う研究の多くは検体入手の比較的容易な不妊医療あるいは産婦人科医療の現場と結びついて実施されている。環境毒性学分野の研究の対象集団としては、不妊外来受診者は曝露と無関係に妊孕性の低下した人が最初から多く含まれるという点で一般人口とは異なり、また、産婦人科外来を受診した妊婦の

配偶者を対象にボランティアを募る方法は、今度は子供をもつ年齢層の妊孕力のある人のみが選択されるため、独身者、子供のいない人、子供をつくる時期を過ぎた人もいる一般人口とは異なった集団とみなされる。これに対し、職域集団を対象に行う研究は、精液採取の問題による参加率の低ささえ解決すれば上記の欠点を回避でき、検討対象となる化学物質への曝露量も高いという、医療機関ベースの研究にはない長所がある。

これまでに発表された職域での精液指標の調査研究は、症例群に関する記述研究によるハザード事例の報告(3, 4)あるいは横断研究(5-26)の手法によるものに大別される（表１）。ごく一部はパネル研究として時間軸を含み、その場合は同一標本の曝露前後における、あるいは曝露頻度の変化にともなう精液指標の変化の有無を検討可能である(7, 18, 19)。曝露が指標悪化のリスクを増強しているかどうかより直接的に明らかにするためには、１つの職域において前向きコホートを設定することが望ましいが、精液指標測定については１回限りの横断研究実施すら容易でなく、今日までに発表されたコホート研究は、曝露作業者が不妊問題を抱えたことで知られる 1,2-ジブロモ-3-クロロプロパン(DBCP)(27)についての発表を除きほとんど存在しない。

職域における研究では、精液指標変化の検出に必要な標本の大きさの確保が最大の難所である。日本国内の職域では近年省力化が著しく進み一事業所あたりの曝露者が減っていて、調査対象人数の限られる場合が少なくない。これは、精液指標に関する調査研究に特有の問題ではないが、曝露者が少ない上に参加率が低い場合は、適切な対照群の設定に難渋し量反応関係の検討が困難となる。曝露量が少なく大きな影響がみこまれない場合は、調査前に参加者数が確定した時点で曝露群と非曝露群の間で有意差の出ないことが予想されることになり、このような場合は調査研究を実施すべきでないという考え方もありうる。しかし、参加者を募る手続きは事業所側の了解を取り付けた後でなければ行えず、「参加率が低い場合には調査は実施しません」という姿勢では、調査への協力の可否を事業所の安全衛生委員会をはじめとする意思決定プロセスにのせて検討してもらうことはむずかしい。また、対象者には参加者個々の健康管理より研究上の関心が優先しているように受け取られる可能性があり、その場合研究参加呼びかけの説得力に欠けるであろう。しかし困ったことに、参加希望者が少ないことが明らかになった段階で申込期間の延長とともに説明をさらに繰り返しても、筆者の経験では精液調査への参加希望者が新たに現れたことはほと

んどなく、参加率が上昇することはまず望めない。

したがって、職域で精液指標調査を行おうとする場合は、分析研究として成り立たないことが判明しても調査を実施する覚悟が必要である。表１に示すように、調査研究への参加率は最大でも３割と見積もるのが現実的であり、対象職域の人数とこの３割という数字が、分析的横断研究として成立するか、また調査全体にどの程度の時間と器材などの資源が必要か検討する上で出発点となる。研究実施にあたってはｐ値や信頼区間の記載を行い、結果の判断を読者に委ねるとともに将来のメタアナリシスに使用できる情報を残すよう努力すべきであるが、不幸にも参加者数がきわめて少ない場合は、各被験者の曝露の状況と精液指標所見の記述研究として調査を行う。その場合も、研究参加者や協力した事業所に対する産業保健サービスとしての側面には変化がないことを忘れるわけにはいかない。

３．事業所の理解と協力

精液採取は結果の研究への利用を前提とする健康診断として実施するが、職域集団を対象とする以上、被験者のインフォームドコンセント取得手続き以前に事業所の理解と協力を得ることが不可欠である。これは精液指標調査研究に特異的な問題ではないが、毒性学的な視点での研究実施に必要な留意点がいくつか存在する。

職域集団における生殖機能の保護は、法的には労働基準法、労働安全衛生法に基づいて行われる。女性については労働基準法第６章の２で、妊産婦等の危険有害業務への就業制限や生理休暇等について定めているが、男性生殖機能保護の視点は法の中にない。一方、労働安全衛生法第 66 条 2 項による特殊健康診断では、現状ではグリコールエーテル類(28)や鉛(13, 29)のように使用頻度が高く、ヒトで精液質への毒性影響が起こりうることが知られる化学物質についても、健康診断項目に生殖機能に関する検査や自覚症状の問診は規定されていない。したがって、精液質の健康管理、すなわち精巣毒性の予防という概念は事業所側にとってなじみがない。調査研究の実施が従業員の健康管理に役立つと考え協力が得られる状況なら理想的であるが、生殖機能はプライバシー性が高く羞恥心も無視できない領域であるために、私生活への介入と考え実施に二の足を踏むのが通常の反応である。

また、事業所側としては、従業員の意思やプライバシーの尊重という側面の

他に、潜在している生殖機能への影響が検出された場合にどのように対処すべきかを考慮する必要がある。その結果、健康障害の発生を疑わせる結果が得られた場合に従業員に対し責任を負えないとの理由で協力を断られたり、事業主が調査研究の意義を正しく認識しても、社内の意思決定過程で産業保健スタッフ、労働組合、法務担当者を含むすべての関係者の合意を最終的に得られなかったりしたことがあった。一方で成功例としては、事業所の了承を得た上で安全衛生委員会に出席する嘱託産業医を訪問し、調査研究内容について説明し理解を得たことにより、当初躊躇していた事業所の姿勢が協力に転じた事例を経験している。

調査の実施方法としては、事業所が主宰する定期健康診断の任意の付加項目として精液検査を含む研究的な健康診断を行う方法に加え、事業所が行う健康診断とは無関係に事業所外に会場を設定し、事業所には個々の従業員への参加協力依頼の橋渡しのみお願いする方法がある。前者は事業所として従業員の参加状況を把握できるが、健康診断結果が悪ければ何らかの対応を迫られる可能性がある。一方、後者の場合は調査研究としての健康診断への参加とその結果の利用は、基本的に参加者が私的に行うものと理解される。どちらの方法を受け入れやすいかは事業所により異なるが、後者を望む場合が多い印象を筆者は受けている。

いずれの実施方法にせよ、毒性学的な視点での調査結果は精液指標に限らず社会の関心事であり、事業所は研究結果への不安を常に持っている。対象化学物質によっては該当企業が限られるため、そのような場合は企業名を匿名にして研究発表を行っても職域が特定される可能性がある。労働安全衛生法に定めのない自主的な健康調査を実施している姿勢が従業員はもとよりその企業に対する地域社会や国民の信頼をより高める方向に働くか、あるいは研究として公表された調査結果がマスコミによりセンセーショナルにとりあげられ、企業や事業内容にダメージを与える方向にむかうかは、研究者の結果発表のしかた、マスコミの報道のしかた、市民運動家の行動、情報を受け取る国民の側の態度によるところが大きいと思われる。職域で毒性学的な研究を行う研究者なら誰でも理解していることであるが、未知の問題点を明らかにする視点だけで調査を行った場合、たとえ健康管理の向上や知見の収集に貢献できても、協力した事業所が途方に暮れる事態が生じうる。主治医が患者に対して責任を負うのと同様に、研究者側の道義的責任として、事業所及び研究参加者の双方に生じる

問題の解決に協力することが求められる。

4．被験者の理解と協力

事業所の了承が得られたとして、対象者の理解と協力を得て精液採取を伴う調査研究への参加率をいかに上げるかが課題である。子供を望むカップルの約10-15%は不妊の問題を抱える(30)が、生殖機能の健康管理という概念は一般的とはいえず、精液を健康診断で提出することには心理的抵抗がきわめて強い。電話での問い合わせ、アンケート、あるいは事業所の安全衛生担当者などを通じてこれまでによせられた疑問や不参加者の声としては、エイズや梅毒その他の性病検査との誤解、検診に参加するのはそのような病気が心配だからではないかと他人に勘ぐられることの心配、精液採取には侵襲的な苦痛を伴うのではという心配、精液検査またはマスターベーションへの抵抗、子供がいるので検査の必要を感じないなどの内容があった。プライバシーの保持に関しては、調査研究への参加の有無を同僚に知られないよう、参加者同士が採精の前後出会わないなどの配慮は常に行っているが、採精室を事業所内に設定した時は職場内で受診の秘密を保つのは事実上不可能であった。ちなみに、参加者の受診時刻の設定においては、採精に要する時間のばらつき（平均15分程度であるが30分以上かかる場合もある）や液化待機時間 30 分、1検体の測定に要する時間（15～30 分)、被験者の動線上で他人と会わないための予備時間を考慮し、最低45～60分は被験者間の間隔をあけるようにしている。

精子数の計測が主要な目的であれば精液採取は自宅で行い持参してもらう方法もありうるが、次章で述べるように分析前の検体取り扱いに留意する必要が生じ煩雑であるほかに、頻度が高くかつ精液所見を悪くすることが知られている精索静脈瘤(31)の診察や精巣容積の測定を、健康診断の一体の流れの中で実施することがむずかしくなる。ちなみに、この生殖器系の診察も参加者の心理的抵抗感を高める要因の1つであり、同じ内容の説明と同じ泌尿器科専門医による診察を実施しても、診察を病院の外来で行った時には比較的問題にならず、事業所内の一室で行った時に説明が足りないとの指摘を受けた経験がある。同じ手続きを踏んで健康診断を行っても、実施場所が事業所内か医療機関内であるかによって参加する側の意識が微妙に変わる可能性は、生殖器を扱う調査研究以外では通常問題にならないだろうと考えられる。

調査研究の目的と意義、参加するメリット、デメリットについて対象者に十