FLOW CYTOMETRY ANALYSIS OF THE CHANGES OF SPERM FUNCTIONS IN YU-CHENG YOUNG MEN PRENATALLY EXPOSED TO POLYCHLORINATED BIPHENYLS AND DIBENZOFURANS

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Introduction

During 1978-1979, over 2,000 of Taiwanese ingested rice oil contaminated with polychlorinated biphenyls (PCBs) and its pyrolytic product, mainly polychlorinated dibenzofurans (PCDFs). This episode was referred to as "Yu-cheng" ("oil-disease" in Chinese) exposure[1]. They have reported more chloracne, hyperkeratosis, abnormal nails, and skin allergy than the background exposure groups and reviewed health effects elsewhere[2]. Among men prenatally exposed to PCBs/PCDFs, increased sperm abnormal morphology, reduced sperm motility and capability of penetrating oocytes[3] were reported. Flow cytometry (FCM) is a tool with great potential for performing both qualitative and quantitative analyses based on simultaneous measurements of structural and functional parameters of individual cells[4]. These measurements are based on light scatter, which reflects cell size and structure, and fluorescence, which can reflect DNA content, enzyme activity, respiration, membrane potential, or membrane integrity depending on the fluorescent dye being used. In combination with fluorescent dyes, we performed concomitantly four well-defined cytofluorometric assays to assess sperm mitochondrial potential (MMP), reactive oxygen species (ROS) generation, and chromatin DNA integrity in semen samples from Yu-cheng young men and their controls.

The aim of this study was to establish correlation between the changes of sperm functions using FCM and the quality of sperm evaluated using conventional light microscopy analysis to evaluate among Yu-cheng young men and control group.

Materials and methods

Overview of Study Design:

A total of 35 young men born to Yu-cheng women exposed to PCBs/PCDFs in 1979-1982 and 15 graduate students from a university in south Taiwan. Including the graduate students, a total of 50 participants agree to complete questionnaires including basic information, lifestyle, and sexual function and donate their semen sample. We measured the parameters of semen quality, such as semen volume, sperm concentration, motility, morphology in according with WHO guideline[5]. All interviews, examinations, and laboratory tests were performed in a blinded fashion. We further analysed sperm MMP, ROS generation and sperm chromatin structure analysis (SCSA) using FCM. Finally, we evaluated the relationships between sperm functions using multiple linear regressions.

FCM Analysis for Sperm MMP, ROS Generation, and SCSA:

The lipophilic cation with dual emission fluorophore JC-1 was used to measure the MMP. When excited by blue light at 488 nm, if the JC-1 molecule remains in its monomeric form. After passing through the mitochondrial membrane, it will exhibit green fluoresce at 530 nm, representing low potential (inactivity or death). If JC-1 molecule transforms into the J-aggregate form, it will exhibit an orange fluoresce at 590 nm, indicating high membrane potential (high activity). Intracellular $O_2^{-\cdot}$ and H_2O_2 levels were measured through the use of fluorescent probe hydroethidine and 2',7'-dichlorofluorescin diacetate using FCM.

The SCSA was used to detect the susceptibility of sperm to in situ acid denaturation of DNA. Acridine orange was used to distinguish stain double-stranded from single-stranded nucleic acids. The metachromatic shift from green to red fluorescence was expressed as αT, a ratio between red and total fluorescence [red/(red 1 green)]. Each sample was calculated and results were expressed as the mean of the αT distribution reflecting the level of sperm with DNA damage. Spermatozoa with abnormal chromatin structure or DNA damage were represented by DNA fragmentation index (DFI).

Results and discussion

Compared with 15 control subjects, the 35 Yu-cheng young men were about the same age, similar in body weight, body mass index, age of first ejaculation or nocturnal emission, frequency of erection per day, abstinence time from ejaculation before study, and alcohol drinking. We found that body height and education were significantly lower and smoking rate was significantly higher among Yu-cheng group compared to the control group (Table 1).

Semen volume, sperm concentration, motility, and normal morphology were all significantly reduced in Yu-Cheng group (Table 2). Sperm abnormal morphology, including head defect and tail defect as well as prevalence rate of asthenozoospermia were significantly increased in Yu-Cheng group (Table 2).

The outcomes of FCM analysis of semen quality were shown in Table 3. No significant differences were found for MMP, sperm H_2O_2 generation, and DFI. The percentage of sperm with excessive $O_2^{\cdot-}$ generation and the intensity level for sperm $O_2^{\cdot-}$ were significantly increased in Yu-cheng group than the control group (Table 3). After adjusting for age, body weight, body mass index, smoking, alcohol drinking and hot bath, it was found that the decrease in sperm motility (β = -0.49, p = 0.0048) and normal morphology (β = -0.32, p < 0.0001) was inversely associated with the percentage of sperm with excessive $O_2^{\cdot-}$ generation, respectively. The percentage of sperm with excessive H_2O_2 generation was found to be inversely correlated with sperm normal morphology (β = -2.59, p = 0.0317) as illustrated in Table 4.

Conventionally, semen quality is typically measured by assessing sperm count, motility, morphology, and vitality. Recently, sperm chromatin DNA integrity, MMP, and ROS generation have also been considered as important parameters for evaluating semen quality, and might be correlated with other measures[6]. An animal study has demonstrated that spermatozoa of PCB 132-exposed offspring produced significantly higher levels of ROS than the controls; ROS induction and sperm-oocyte penetration rate reduction were dose-related[7]. Some epidemiological studies suggested that human dietary PCB exposure might have a negative impact on the sperm chromatin integrity of adult males[8, 9]. Rignell-Hydbom et al (2005)[10] performed also study among 176 Swedish fishermen with low and high consumption of fatty fish. A significantly lower % DFI was found in the lowest PCB 153 quintile (<113 ng/g lipid) compared with the other quintiles. Many of the endocrine disrupters have been linked to adverse effects on either embryonic development or reproductive function in humans and wildlife[12-14]. In conclusion, spermatozoa of PCBs/PCDFs prenatally exposed Yu-cheng young men have decrease of semen volume, sperm concentration, motility, and increasing sperm ROS generation. It might be associated with sperm count, motility and morphology via sperm excessive ROS generation. The detail mechanisms still need to be clarified.

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Table 1. Comparisons of demographic characteristics between Yu-cheng group (n = 35) and control group (n = 15).

Characteristics	Control (n=15)	Yu-cheng (n=35)	*p*-value
Age (years)	25.3±3.4	25.0±4.3	0.7964
Body height (cm)	175.7±5.5	172.0±4.8*	0.0206
Body weight (kg)	67.1±7.4	70.5±12.3	0.3267
BMI (kg/m^2)	21.7±2.3	23.8±4.0	0.0687
Education (years)	18. 0±0.0	14.3±2.0*	<0.0001
Age of first ejaculation or nocturnal emission (years)	14.3±1.8	15.0±2.1	0.2639
Frequency of erection (per day)	2.9±1.8	2.9±4.0	0.9779
Abstinence from ejaculation before study (days)	4.3±3.1	4.5±5.6	0.8960
Cigarette smoking			
Yes	1(6.7%)	17(48.6%)*	0.0021
No	14(93.3%)	18(51.4%)	
Alcohol drinking			
Yes	2(13.3%)	7(20.0%)*	0.5651
No	13(86.7%)	28(80.0%)	

Data are expressed as mean±S.D. (Standard deviation) or number (%). *p<0.05 as compared with control group.

Table 2. Conventional analysis of the changes of semen quality between Yu-cheng group (n = 35) and control group (n = 15).

Characteristics	Control (n=15)	Yu-cheng (n=35)	*p*-value
Semen volume (mL)	3.8 ± 1.6	2.4 ± 1.0*	0.0004
Sperm count (10^6 sperm/ml)	162.4 ± 107.9	96.0 ± 104.2*	0.0466
Sperm motility (%)	65.2 ± 10.0	46.6 ± 3.0*	0.0004
Normal morphology (%)	44.9 ± 7.7	17.7 ± 7.5*	< 0.0001
Head defects (%)	13.9 ± 1.7	19.99 ± 1.1*	0.0040
Midpiece defects (%)	12.8 ± 1.2	13.9 ± 0.8	0.4422
Tail defects (%)	28.4 ± 2.0	48.7 ± 1.3*	< 0.0001
Oligozoospermia [yes, (%)]	1(6.7)	5(14.29)	0.4250
Asthenozoospermia [yes, (%)]	0(0)	17(48.6)*	< 0.0001

Data are expressed as mean±S.D. (Standard deviation) or number (%). *p<0.05 as compared with control group.

Table 3. Flow cytometry analysis of the changes of semen quality between Yu-cheng group (n = 35) and control group (n = 15).

Characteristics	Control (n=15)	Yu-cheng (n=35)	*p*-value
Normal MMP (%)	15.4±10.5	18.6±14.2	0.4470
Sperm $O_2^{-\cdot}$ (%)	3.0±2.3	45.0±2.6*	<0.0001
Sperm $O_2^{-\cdot}$ (AU)	36.2±7.3	356.8±164.5*	<0.0001
Sperm H_2O_2 (%)	0.9±0.8	1.6±1.6	0.1109
Sperm H_2O_2 (AU)	22.7±5.1	23.8±7.1	0.5876
DFI (AU)	345.9±9.0	338.5±10.1	0.4998

Data are expressed as mean±S.D. (Standard deviation). *p<0.05 as compared with control group; MMP：Mitochondrial membrane potential; AU: stain intensity; DFI: DNA fragmentation index.

Table 4. The multiple regression analysis of semen quality characteristics on sperm ROS generation (%) after adjusting for age, body height, body weight, BMI, education years, smoking, alcohol drinking, and hot bath among Yu-cheng group and control group.

Characteristic	Sperm $O_2^{-\cdot}$ (%)		Sperm H_2O_2 (%)	
	β(SE β)	*p*-value	β(SE β)	*p*-value
Sperm count (10^6 sperm/ml)	-0.95(0.86)	0.4809	-19.65(11.91)	0.3823
Sperm motile (%)	-0.49(0.12)	0.0048	-2.73(1.91)	0.2623
Normal morphology (%)	-0.32(0.09)	<0.0001	-2.59(1.30)	0.0317
DFI (AU)	0.02(0.28)	0.7225	2.06(3.99)	0.7236
MMP (%)	-0.18(0.10)	0.2996	-0.01 (1.49)	0.6195

SE β: Standard error of β.