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Exposure to acrylonitrile induced DNA strand breakage and sex chromosome aneuploidy in human spermatozoa

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Abstract

To explore acrylonitrile (ACN)-induced DNA strand breakage and sex chromosome aneuploidy in human spermatozoa, semen parameters were examined among 30 acrylonitrile-exposed workers according to WHO laboratory manual for the examination of human sperm. DNA strand breakage of sperm cells was investigated among 30 ACN-exposed workers using single cell gel electrophoresis (SCGE). The frequency of sex chromosome aneuploidy in sperm cells was analyzed among nine ACN-exposed workers using fluorescence in situ hybridization (FISH). The geometrical mean of sperm density was 75×10^6 ml^{-1} in exposure group, significantly lower than 140×10^6 ml^{-1} in the control. The geometrical mean of sperm number per ejaculum was 205×10^6 in exposure group, significantly lower than 280×10^6 in the control. The rates of comet sperm nuclei were 28.7% in exposure group, significantly higher than 15.0% in the control. Mean tail length was 9.8 μm in exposure group, longer than 4.3 μm in the control. The frequency of sex chromosome disomy was 0.69% in exposure group, significantly higher than 0.35% in the control. XY-bearing sperm was the most common sex chromosome disomy, with an average rate of 0.37% in exposure group, and 0.20% in the control. XX- and YY-bearing sperm accounted for an additional 0.09 and 0.23% in exposure group, and 0.05 and 0.10% in the control. The results indicate that ACN affect semen quality among ACN-exposed workers. ACN or its metabolites could induce reproductive defects as an in vivo multipotent genotoxic agent by inducing DNA strand breakage and sex chromosome non-disjunction in spermatogenesis.

Keywords: Acrylonitrile; Sperm; DNA damage; Chromosome; Aneuploidy

1. Introduction

Acrylonitrile (ACN) is a chemical used extensively in the production of plastics, resins, synthetic fibers and rubbers. Many studies indicated that ACN is a probable human carcinogen based on limited evidence in humans and rather extensive evidence in laboratory animals [1,2]. ACN also induces morphological transformation concomitant with an increase in the formation of oxidized DNA in Syrian Hamster Embryo (SHE) cells in a dose-dependent manner [3]. ACN-induced genotoxic effects have been reported in somatic and germ cells in laboratory animals, and also in lymphocytes of ACN-exposed workers [4–9]. A recent research showed that ACN induced 8-OHdG increases in brain tissue in male SD rats [10].

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ACN is teratogenic in laboratory animals (rat, hamster) at high doses when maternal toxicity already is manifest. ACN have been demonstrated to induce embryotoxic effects in rat [11,12]. ACN-induced embryotoxic and teratogenic effects have also been found in ACN-exposed workers [13]. According to recent environmental teratologic epidemiological study in inhabitants living in the surrounding region of an acrylonitrile factory, three congenital abnormalities (pectus excavatum, undescended testis and clubfoot) in 46,326 infants showed significant time-space clusters in the study region. There was a decrease in risk of undescended testis with increasing distance from the acrylonitrile factory [14]. Therefore, women not professionally exposed would appear to be at risk of teratogenic effects due to ACN.

On the other hand, although there were a few literature concerning ACN-induced male reproductive effects in laboratory animals, whether ACN induces male reproductive toxicity in ACN-exposed workers is so far not clear. To explore ACN-induced male reproductive toxicity in ACN-exposed workers, semen parameters were examined among 30 ACN-exposed workers according to WHO laboratory manual for the examination of human sperm. DNA strand breakage of sperm cells was then investigated among 30 ACN-exposed workers using single cell gel electrophoresis (SCGE). Finally, the frequency of sex chromosome aneuploidy in sperm cells was analyzed among nine ACN-exposed workers using fluorescence in situ hybridization (FISH).

2. Materials and methods

2.1. Materials

Low melting point agarose (LMPA) and normal melting point agarose (NMPA) were obtained from Life Technologies, Inc. (Gaitherburg, MD, USA). An alpha satellite chromosome X specific DNA probe labeled with biotin (DXZ1) and an alpha satellite chromosome Y specific DNA probe labeled with digoxigenin (DYZ3) were purchased from Oncor. Dithiothreitol (DTT), salmon sperm DNA, formamide, streptavidin-Cy3, mouse anti-digoxigenin, biotinylated goat anti-streptavidin, rabbit anti-mouse-FITC, goat anti-rabbit-FITC, RNase A and proteinase K were from Sigma (St. Louis, MO, USA).

2.2. Sample collection

Thirty sperm donors whose ages ranged from 25 to 30 years were recruited from the ACN-exposed workers. All subjects were recruited from the same chemical plant. This factory had been put into production for only 2.8 years. So all subjects' exposure-time was 2.8 years. The mean concentration of ACN was $0.8 \pm 0.25\ mg/m^3$ at operation sites. Another 30 sperm donors whose ages ranged from 24 to 35 years were recruited from the general population who had no history of exposure to ACN. None of the subjects had chronic disease and exposure to chemotherapy or radiotherapy. All of subjects were non-smokers and non-regular drinkers. All subjects were asked to collect their semen at a small house by masturbation into a sterile wide-mouth and metal-free plastic container after 3 days of abstinence. Semen samples were incubated at 37 °C for about 30 min to allow the samples to liquefy. Conventional semen analyses for density, sperm number per ejaculum, semen volume, viability, motility, morphology were performed according to WHO guidelines for the examination of human sperm [15]. Semen samples were aliquotted into several eppendrof tubes and kept at −70 °C for subsequent experiments.

2.3. Single-cell gel electrophoresis

The single-cell gel electrophoresis assay was conducted as described by Singh and Stephen [16] with little modification for this study. On the day of experiment, an aliquot of sperm sample was quickly thawed in a 37 °C water bath and used immediately. One hundred microliter of 0.75% normal melting point agarose was placed on a fully frosted slide. The slides were covered with a coverglass and kept at room temperature for at least 5 min. After semen was thawed, sperm was washed twice with PBS. Six microliter of the freshly prepared suspension of cells (about 20,000 sperm cells/ml) was mixed with 54 μl of 0.75% low melting point agarose (LMPA) at 37 °C. After removing the first layer coverglass, 60 μl cells and LMPA mixture was layered onto the first layer of agarose. The slides were put in the fridge for 8 min. Removing the

coverglass, another layer of 120 μl of 0.75% LMPA was then layered on top of the cells, and the slides were put in the fridge for another 8 min. Following procedures were undergone in proper order to pretreat the sperm nuclei. Firstly, the slides were immersed in a cold lysing solution containing 2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris, 1% sodium lauryl sarcosine and, 1% Triton X-100, pH 10 at 4 °C for 1 h to lyse the sperm cells. Secondly, the slides were incubated in a solution of 2.5 M NaCl, 5 mM Tris, 0.05% lauryl sarcosine sodium salt, pH 7.4, with 10 mM DTT at 4 °C for another 1 h to decondense the DNA in the sperm nuclei. Thirdly, the slides were incubated in a solution of 2.5 M NaCl, 5 mM Tris, 0.05% lauryl sarcosine sodium salt, pH 7.4 with 10 μg/ml RNase A at 37 °C for 4 h to remove RNA in sperm cell nuclei. Lastly, the slides were incubated in a solution of 2.5 M NaCl, 5 mM Tris, 0.05% lauryl sarcosine sodium salt and pH 7.4 with 200 μg/ml proteinase K at 37 °C for another 15 h to digest the protein. Slides were placed in a horizontal electrophoresis tank side by side and equilibrated with 300 mM sodium acetate, 100 mM Tris, pH 10 at 4 °C for 20 min. Electrophoresis was performed at 12 V (0.46 V/cm) and 100 mA at 4 °C for 1 h. After slides were neutralized in 0.4 M Tris–HCl buffer (pH 7.0) for at least 5 min, 50 μl of 15 μg/ml EB was added onto each slide to stain the sperm nuclei. Sperm nuclei were viewed with a Nikon epifluorescence microscope at 400× magnification using blue light excitation. Three hundred sperm nuclei were counted each slide.

2.4. Fluorescence in situ hybridization

Samples were thawed and washed for three times in 0.01 M Tris–0.09% NaCl buffer. Two microliter of sperm suspension was smeared on a 1 cm^2 area of a slide and allowed to air dry at room temperature for at least 48 h. Sperm nuclei were decondensed in 10 mM dithiothreitol (DTT) solution for 30 min at room temperature, followed by treatment in 10 mM lithium diiodosalicylate (LIS), 1 mM DTT in 0.1 M Tris for 0.5–3 h at room temperature. Slide was rinsed in 2× SSC solution twice. Probe mix was prepared by mixing 1 μl of DXZ1 probe, 1 μl of DYZ3 probe, 1 μl of salmon sperm DNA and 20 μl of MM2.1. Probe mix was heated at 71 °C for 5 min, and then snap cooled in an ice water bath for at least 5 min. Sperm slides were denatured in a solution of 70% formamide, 2× SSC at 71 °C for 2.5 min, and then snap cooled and dehydrated in 70, 80 and 100% ethanol at −20 °C for 2 min each, and air dried and prewarmed to 37 °C on a slide warmer. Four microliter of probe mix was pipetted onto the slide area over the sperm. Slide was coverslipped, sealed with rubber cement, and then incubated in dark, humidified container at 37 °C for 20 h. Slide was washed twice in 50% formamide at 45 °C for 7.5 min, in 2× SSC solution at 37 °C for 4 min, and rinsed briefly in a Coplin jar containing PBD. Four microliter of PNM buffer was applied and covered with a parafilm coverslip. Slide was incubated in the dark at room temperature for 10 min. Following procedures were undergone in proper order to detect and amplify the signals: firstly, to apply 4 μl of streptavidin-Cy3 (10 μg/ml) mixed with mouse anti-digoxigenin, cover with parafilm coverslip and incubate at 37 °C in a humidified chamber for 30 min. Secondly, to apply 4 μl of biotinylated goat anti-streptavidin (10 μg/ml) mixed with rabbit anti-mouse-FITC, cover with parafilm coverslip and incubate at 37 °C in a humidified chamber for 30 min. Thirdly, to apply 4 μl of strepavidin-Cy3 (10 μg/ml) mixed with goat anti-rabbit-FITC, cover with parafilm coverslip and incubate at 37 °C in a humidified chamber for 30 min. Lastly, to apply 4 μl of DAPI (0.5 μg/ml), cover with parafilm coverslip and incubate in a dark humidified chamber for 10 min. Slide was viewed under 1000× magnification on an epifluorescence fitted with DAPI/FITC/Rhodamine triple-band-pass filter set. Slides were used for counting when hybridization efficiency was at least 95%. About 10,000 sperm nuclei were counted for each donor. A sperm with a red signal was X-bearing chromosome sperm. A sperm with a green signal was Y-bearing chromosome sperm. A sperm was considered to be XX-, YY- or XY-bearing chromosome sperm when two red, two green or a red and a green signals were clearly positioned within the sperm head, comparable in brightness and size, and at least one domain apart. Certain populations of sperm nuclei were eliminated from scoring: overlapping nuclei and disrupted nuclei with indistinct margins; very large nuclei with diffused chromatin, possibly due to overdecondensation and very small nuclei with no signals, possibly due to underdecondensation.

2.5. Statistical analysis

The conventional semen parameters, the rate of comet sperm and frequency of sex chromosome aneuploidy in sperm cells were analysed using the SPSS for Windows (Version 10.0).

3. Results

First, conventional semen analyses for semen volume, density, sperm number per ejaculum, viability, motility, morphology were performed according to WHO guidelines for the examination of human sperm. The results are shown in Table 1. The results showed that the geometrical mean of sperm density was $75 \times 10^6\,ml^{-1}$ in exposure group, significantly lower than $140 \times 10^6\,ml^{-1}$ in the control. The geometrical mean of sperm number per ejaculum was 205×10^6 in exposure group, significantly lower than 280×10^6 in the control. No significant difference was observed in semen volume, sperm motility, viability, or morphological defects between exposure group and the control.

Next, DNA strand breakage was detected in 30 ACN-exposed workers using SCGE. The mean rate of comet sperm was 28.7%, significantly higher than 15.0% in the control. The degree of DNA damage was graded visually into five categories according to the amounts of DNA in the tail: Grade 0, no damage, <5%; Grade 1, low level damage, 5–20%; Grade 2, medium level damage, 20–40%; Grade 3, high level damage, 40–95%; Grade 4, total damage, >95%. Tail length of comet sperm nuclei was measured using an eyepiece micrometer. The results are shown in Table 2. Results showed that mean comet tail length was $9.8 \pm 3.7\,\mu m$ in exposure group, significantly longer than $4.3 \pm 2.3\,\mu m$ in the control.

Last, the frequency of sex chromosome aneuploidy in sperm cells was analyzed among nine ACN-exposed workers using FISH. The results are shown in Fig. 1 and Table 3. Seven hundred and ninety-three sex chromosome nullisome and 633 sex chromosome disome were determined among 91,015 sperm nuclei in exposure group, compared with 445 sex chromosome nullisome and 260 sex chromosome disome among 74,679 sperm nuclei in the control. The frequency of sex chromosome disome in sperm nuclei was 0.69% in the exposure group, significantly higher than 0.35% in the control. XY-bearing sperm was the most common disomic sperm, with an average rate of 0.37% in the exposure group, and 0.20% in the control. XX- and YY-bearing sperm accounted for an additional 0.09 and 0.23% in the exposure group, and 0.05 and 0.10%

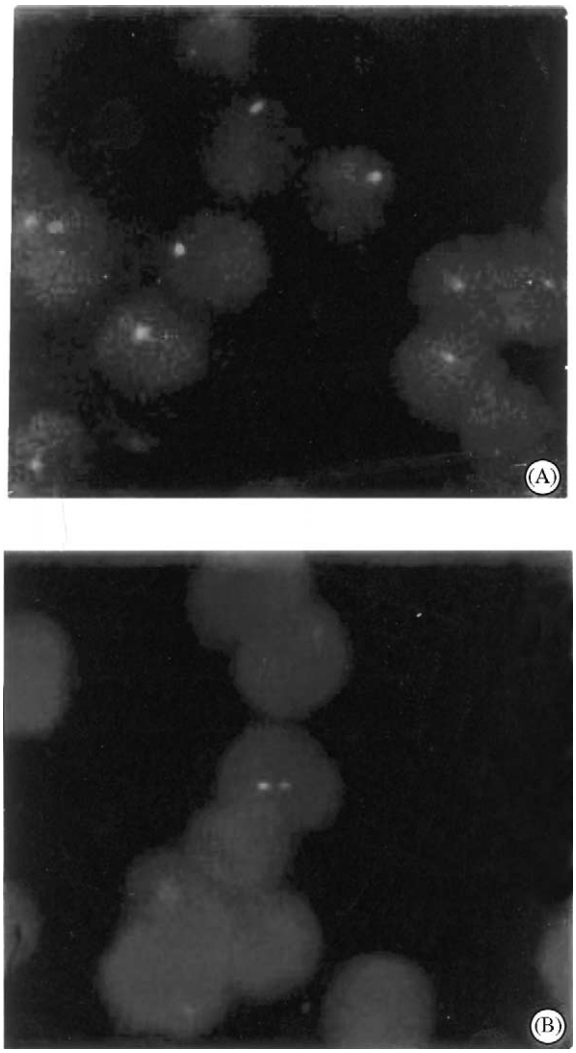

Fig. 1. Photomicrographs of disomy in human spermatozoa. (A) Normal spermatozoa (a sperm with a red signal was X-bearing chromosome sperm. A sperm with a green signal was Y-bearing chromosome sperm); (B) YY-bearing chromosome sperm in which two green signals were clearly positioned within the sperm head.

Table 1
Comparison of conventional semen parameters between acrylonitrile-exposed workers and the control

	Exposure (geometrical means ± *s*)	Control (geometrical means ± *s*)
Volume (ml)	2.5 ± 0.8	2.2 ± 0.7
Density ($\times 10^6$ ml^{-1})	75 ± 12**	140 ± 27
Sperm per ejaculum ($\times 10^6$)	205 ± 120*	280 ± 112
Viability (%)	61 ± 12	59 ± 8
Motility (%, Δ)	60 ± 10	59 ± 8
Abnormality (%)	48 ± 11	49 ± 9

* $P < 0.05$.
** $P < 0.01$ compared with control group.

Table 2
Comparison of tail length and rates of comet sperm between acrylonitrile-exposed workers and the control

Groups	*n*	Counted sperm	Tail length (means ± *s*)	Rates of comet sperm				
				G1	G2	G3	G4	Total
Exposure	30	9000	9.8 ± 3.7**	5.0	8.0	6.7	9.0	28.7**
Control	30	9000	4.3 ± 2.3	4.0	3.0	4.0	4.0	15.0

** $P < 0.01$ compared with control group.

Table 3
Comparison of frequencies of sex chromosome aneuploidy between acrylonitrile-exposed workers and the control

Groups	Counted sperm	Nullisome		XX sperm		YY sperm		XY sperm		Disome	
		No.	%	No.	%	No.	%	No.	%	No.	%
Exposure	91015	793	0.87**	86	0.09*	213	0.23**	334	0.37**	633	0.69**
Control	74679	445	0.60	36	0.05	77	0.10	147	0.20	260	0.35

* $P < 0.05$.
** $P < 0.01$ compared with control group.

in the control. There were significant differences in the frequencies of XX-, YY- and XY-bearing sperm between exposure group and the control.

4. Discussion

ACN-induced male reproductive toxicity had been found in laboratory animals. Daily oral administration of ACN (10 mg/kg) to mice for a period of 60 days caused a significant decrease in the activities of testicular sorbitol dehydrogenase and acid phosphatase, and an increase in that of lactate dehydrogenase and beta-glucuronidase. Histopathological studies revealed degeneration of the seminiferous tubules. A decrease in the sperm counts of the epididymal spermatozoa was also observed in the animals of the ACN-exposed group [7]. To explore whether ACN induced male reproductive toxicity in ACN-exposed workers, we examined semen conventional parameters for density, sperm number per ejaculum, semen volume, viability, motility, morphological defects according to WHO guidelines. Thirty sperm donors whose ages ranged from 25 to 30 years were recruited from the ACN-exposed workers who had been exposed to 0.8 mg/m^3 of ACN for 2.8 years. None of the subjects had chronic disease and exposure to chemotherapy or radiotherapy. All of subjects were non-smokers and non-regular drinkers. The results showed that sperm density and sperm number per ejaculum were significantly lower in exposure group than in the control. These results indicated that ACN may lead to poor

semen quality in workers who had been exposed to less than 2 mg/m^3 of ACN.

The mechanism of ACN-induced sperm quality decreases was so far not clear. According to Ahmed's report, covalent binding of acrylonitrile to testicular tissue DNA was observed in the testes of rats following a single oral dose (46.5 mg/kg) of ACN. Further, a significant decrease in DNA synthesis was found at 0.5 h after treatment of a single oral dose (46.5 mg/kg) of ACN. At 24 h following ACN administration, testicular DNA synthesis was severely inhibited. Testicular DNA repair was increased 1.5-fold at 0.5 h and more than 3.3-fold at 24 h following treatment with ACN [17]. These results suggest that ACN can permeate blood–testis barrier and is a genotoxic agent in testicular tissue. On the other hand, one major pathway of ACN metabolism is through glutathione (GSH) conjugation. Extensive utilization and depletion of GSH, an important intracellular antioxidant, by ACN may lead to cellular oxidative stress. Recently, acrylonitrile has also been found to cause or enhance oxidative DNA damage in glial cells [18,19]. Single cell gel electrophoresis (SCGE) is a sensitive and rapid method for direct visualization of DNA strand breakage in individual cells and has been used extensively in somatic cells [20]. In present study, SCGE was used to investigate ACN-induced DNA strand breaks in sperm cells among ACN-exposed workers. Results showed that the rate of comet sperm nuclei was 28.7% in exposure group, significantly higher than 15.0% in the control. Mean tail length was 9.8 μm in exposure group, longer than 4.3 μm in the control. These results indicated that ACN induced the in vivo DNA damage in human sperm cells, as did it in somatic cells [4,18,19,21]. Some studies showed that a significant inverse correlation exists between DNA damage and sperm density, and between DNA damage and sperm number per ejaculum [22–24]. Therefore, we estimate that ACN may lead to decreases of sperm density and sperm number per ejaculum by inducing DNA strand breakage in human sperm cells. Our study found that sperm motility, viability and morphological defects were not affected by ACN exposure. The results were consistent with those by Ni's study in which no significant correlations were observed between oxidative DNA damage in human sperm cells and sperm motility, viability and morphological defects [22].

An earlier epidemiological investigation on reproductive outcomes of 534 ACN-exposed subjects' wives had found that the relative risk was 4.73 (95% CI: 1.35–16.53) for stillbirth, 3.89 (95% CI: 1.32–11.51) for birth defects and 2.46 (95% CI: 1.23–4.90) for spontaneous abortion [25]. Based on the literature, aneuploidy transmitted via germ cells is a major contributor to infertility, spontaneous abortion, stillbirths and infant death. The incidence of trisomy is 0.3% among newborns, of which 50% involve sex chromosome. The overall frequency of trisomy is about 4.3% among stillbirths, over 10-fold that for newborns. About 35% of numerical abnormalities are observed among spontaneous abortive embryo, representing 100-fold increase over that observed in newborns [26]. In this study, FISH was used to investigate the in vivo ACN-induced sex chromosome aneuploidy in ejaculated spermatozoa among ACN-exposed-workers. A total of 91,015 sperm nuclei in nine ACN-exposed workers were investigated. The rate of sex chromosome disomy in sperm cells was 0.69% in exposure group, significantly higher than 0.35% in the control. XY-bearing sperm was the most common sex chromosome disomic cells, with an average rate of 0.37% in exposure group, significantly higher than 0.20% in the control. XX- and YY-bearing sperm accounted for an additional 0.09 and 0.23% in exposed group, significantly higher than 0.05 and 0.10% in the control. The results were consistent with those by Osgood's study in which ACN-induced sex chromosome loss was observed in *Drosophila* [27]. These observations suggest that ACN may induce sex chromosome non-disjunction in spermatogenesis. ACN-induced sex chromosome aneuploidy may be related to stillbirth, birth defects and spontaneous abortion among ACN-exposed workers' wives. Furthermore, aneuploidy in germ cells is also a major cause of congenital malformation, mental retardation, behavioral abnormalities and human genetic diseases. Sex chromosome aneuploidy in humans is predominant of paternal meiotic origin. The fraction of affected offspring in which the extra chromosome is of paternal origin is estimated to be 44% for 47 XXY and 100% for 47 XYY [28]. Among 45 X cases, about 77% of newborns and 83% of spontaneous abortions are due to lack of paternal chromosome [29]. The close correlations of sex chromosome non-disjunction in spermatogenesis with

stillbirth, birth defects and spontaneous abortion, congenital malformation, mental retardation, behavioral abnormalities and human genetic diseases indicate the importance of investigation into sex chromosome aneuploidy aberration in a large sample of ACN-exposed workers.

In summary, present data demonstrated that ACN lead to decrease in sperm quality among ACN-exposed workers. ACN or its metabolites can induce reproductive defects as an in vivo multipotent genotoxic agent by inducing DNA strand breakage and sex chromosome non-disjunction in spermatogenesis.

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