Chemosphere 80 (2010) 972–977

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Y Chromosome microdeletion and altered sperm quality in human males with high concentration of seminal hexachlorocyclohexane (HCH)

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ARTICLE INFO

Article history:
Received 20 January 2010
Received in revised form 23 April 2010
Accepted 26 May 2010
Available online 18 June 2010

Keywords:
Hexachlorocyclohexane
Yq microdeletion
Semen quality
Sperm count
Sperm motility

ABSTRACT

Recent studies have shown Y chromosome microdeletions associated with male infertility. The factors responsible for Y chromosome microdeletions in spermatozoa remain unresolved. However, the environmental pollutants are known to damage DNA in differentiating and maturing germ cells in the male reproductive tract. Therefore, the aim of this study was to investigate the effects of seminal hexachlorocyclohexane (HCH) and its isomers, an environmental pollutant, in 50 fertile and 50 infertile males in relation to semen quality and the incidence of Y chromosome microdeletion in azoospermic factor (*AZF*) region. As compared to control, an increased HCH level and significantly decreased semen quality were observed in the infertile males. A positive significant association was found between sperm count with α-HCH and β-HCH in the infertile males. A negative significant association was observed between sperm counts with γ-HCH in asthenospermia patients and with β-HCH and total HCH in oligo-asthenospermic patients. Out of 100 males studied, we found 10 patients with Yq deletion in *AZFa* and *AZFc* regions. Subdivision of infertile group revealed a deletion incidence of 61.5% in azoospermic patients, 11.1% in oligospermic patients and 16.6% in oligo-asthenospermic patients. The presence of Yq deletion in azoospermic patients with a significant mean difference of β-HCH and total HCH in relation to reduced semen quality seem to corroborate with the mutagenic activity of HCH. The results of this study indicated the susceptibility of male germ line to mutagenic potential of HCH which is an acknowledged risk factor leading to spermatogenic failure.

1. Introduction

In the past few decades, a remarkable drop in human male fertility has been observed due to environmental stress all over the world. The increased incidence of male infertility is gaining wide attention towards the progressive decline in semen quality (particularly in sperm counts) over the past half a century (Carlsen et al., 1992; Auger et al., 1995). The epidemiological evidence suggested that the reduced semen quality is a general cause of approximately 25% of infertility among couples (Templeton, 1995). The aetiology of reduced semen quality is not well understood. However, DNA fragmentation appears to adversely affect the semen quality, especially sperm counts, morphology and motility (Irvine et al., 2000; Muratori et al., 2000; Shen and Ong, 2000). Moreover, several studies have shown a negative correlation between the stability of sperm DNA and the fertilizing capacity of spermatozoa (Aitken et al., 1998; Evenson et al., 1999; Host et al., 2000).

The integrity of DNA is affected by oxidative stress when the production of reactive oxygen species (ROS) overwhelms antioxidant defense mechanism (Halliwell et al., 1992). The elevated level of testicular ROS may influence the Y chromosome microdeletion and DNA damage, which may play a vital role in reproductive dysfunctions (Barroso et al., 2000; Wang et al., 2003; Said et al., 2005). The possible sources of ROSs include abnormal spermatozoa, lifestyle, environmental and occupational exposure to toxic pollutants (Padron et al., 1997; Aitken and Krausz, 2001). The presence of xenobiotics induces redox cycling by spermatozoa and generates toxic free radicals. Hexachlorocyclohexane (HCH), an organochlorine pesticide can alter the normal regulatory function of the endocrine system and induces oxidative stress in the testis (Samanta and Chainy, 1997). The induction of oxidative stress impairs the testicular functions in adult age as a consequence of some permanent lesions in response to HCH exposure during critical stages of sexual maturation (Samanta et al., 1999). HCH exists in five stable isomers encompassing α-HCH, β-HCH, γ-HCH, δ-HCH and ε-HCH (Breivik et al., 1996). Relatively, fewer studies were conducted to address the impact of HCH on reproductive health. Therefore, there is a need to screen the genetic damage in sperm DNA to establish its correlation with male infertility, if any, which may be a valuable biomarker of environmental exposure.

The genetic variation that underpins the evolutionary process appears predominantly in the male germ line because of the inability of the haploid genome to deploy recombination repair in

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0045-6535/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.chemosphere.2010.05.047

retrieving the lost genetic information. (Agulnik et al., 1997). The long arm of the Y chromosome is particularly susceptible to microdeletions, which is associated with failure of spermatogenesis (Tiepolo and Zuffardi, 1976; Simoni et al., 1998). The molecular analysis of Y chromosome revealed a de novo microdeletion at one of the three close subregions of azoospermia factor, labeled *AZFa*, *AZFb* and *AZFc* (Kobayashi et al., 1995; Vogt et al., 1996). These three non-overlapping regions of the Y chromosome have been identified in severe oligospemic and azoospermic patients (Vogt et al., 1996). Some candidate genes are recognized in these regions (*DFFRY* for *AZFa*, *RBM* for *AZFb* and *DAZ* for *AZFc*) and are widely used for Y chromosome microdeletion screening (Ma et al., 1993; Reijo et al., 1995; Chai et al., 1997; Brown et al., 1998). The incidence of Y chromosome microdeletions with the infertile phenotype varies widely from 1% (Van der Ven et al., 1997) to 55% (Foresta et al., 1998). The factors responsible for microdeletions of Y chromosome in spermatozoa are still unknown but the level of oxidative stress experienced by germ cells during their differentiation may be one of the controlling aspects of a central reproductive problem.

A special concern for human health hazards arises from the exposure of hazardous waste and dumping sites of HCH-waste from lindane manufacturing plant (Indian Pesticide Limited) at Lucknow (India) which show the elevated levels of HCH in the environment. The recent studies on different human samples viz semen, blood, tissues and milk and in the environmental segment viz. soil, water and vegetables in Lucknow showed the higher residual HCH as compared to other developed countries (Raizada, 1996; Prakash et al., 2004; Mathur et al., 2005; Ahamed et al., 2006; Pant et al., 2007). Therefore, major interest of the present study was to investigate the reproductive toxicity of HCH by measuring the semen quality and Yq microdeletions in the fertile and infertile subjects from Lucknow and its adjoining areas.

2. Materials and methods

2.1. Sample collection and experimental design

We selected 100 male counterparts of couples, attending the outpatient infertility clinic for the suspected infertility at Krishna Medical Center and Makkar Medical Center at Lucknow. Semen samples from all subjects were collected into a wide mouthed plastic sterilized container after 3–5 d of sexual abstinence. After checking the semen quality immediately in the pathology at the infertility clinic, all the samples placed in the icebox were carried to the laboratory for further studies. Based on sperm counts and motility, the subjects were categorized into fertile group and infertile group as shown in Table 2. The control group consists of 50 fertile men whose sperm counts and motility was $>20 \times 10^6$ mL^{-1} and >50% respectively based on WHO (1999) criteria and whose partner had conceived spontaneously within 1 year at the same centers. The infertile group consists of 50 infertile men and subcategorized as oligospermia (sperm concentration $<20 \times 10^6$ mL^{-1}), asthenospermia (<50% motile sperm), oligoasthenospermia (a combination of the two criteria), and azoospermia (no sperm).

Informed consents were obtained from all the participants and each subject was asked to fill-in an extensive questionnaire regarding his occupation, residence, socioeconomic status, diet, smoking habits, pesticide exposure, intake of any ayurvedic, allopathic, homeopathic or traditional medicines and any medical and surgical history. Subjects with past medical history mainly of testicular dysfunction, urogenital abnormality, mumps, tuberculosis, thyroid dysfunction, or surgical operation, using drugs known to affect gonadal function were excluded from the study.

2.2. Semen analysis

Based on WHO (1999) guidelines, semen analyses of all fertile and infertile men were carried out. The evaluation included liquefaction time, colour, odour, pH, viscosity, sperm motility, sperm concentration and presence of pus or epithelial cells. Sperm concentration was determined by an improved Neubauer haemocytometer and sperm motility was assessed by direct observation under a microscope.

2.3. DNA isolation and screening of Y chromosome microdeletion

Genomic DNA was isolated from sperm cells following phenol-chloroform extraction method. The screening of Yq microdeletion was carried out in patients with normal karyotype by amplifying 28 different Sequence-Tagged Sites (STS) marker (Premi et al., 2008) corresponding to the three *AZF* loci (*AZFa*- sY78, sY81, sY84, sY88, sY95, sY746, sY1064, sY1065, sY1066, sY1180, sY1182, sY1184, sY1186; *AZFb*- sY117, sY124, sY125, sY127, sY129, sY131; *AZFc*- sY279, sY579, sY1161, sY1190, sY1191, sY1197, sY1201 sY1206 sY1258). In the events of detecting deletion with primer, the PCR assay was repeated thrice for confirmation.

2.4. Estimation of HCH concentration

Estimation of seminal HCH was carried out following the standard protocol with slight modifications (El-Salem et al., 1982). One milliliter of seminal plasma was homogenized with 5 mL of 1:1 *n*-hexane and formic acid and HCH was extracted with 5 mL, 3 mL and 2 mL of *n*-hexane by shaking it at room temperature for 1 h, 30 min, and 15 min, respectively. The extracted sample was evaporated up to 1 mL volume and cleaned with 5 mL concentrated sulphuric acid by centrifugation at 2000 rpm and 4 °C for 5 min in a graduated glass centrifuge tube. The cleaned extract was again concentrated over a rotary evaporator and finally transferred to a 1 mL volumetric flask and made up to 1 mL volume with *n*-hexane for gas liquid chromatography (GLC) (SHIMADZU-17A). The external standards of different isomers of HCH and the final HCH extract from semen samples were applied on GLC with the following conditions – sample injection: temperature 220 °C, pressure 56 kPa, total flow 11 mL min^{-1}, column flow 0.9 mL min^{-1}, linear velocity 23.7 cm s^{-1}, purge flow 130 mL min^{-1}, split ratio 10, column: BPX-50, length 30 m, inner diameter 0.32 mm, film thickness 0.25 μm, temperature 200 °C, equilibration time 0 min, max temp 300 °C, detector: Ni63 ECD, temperature 250 °C, range – 1, current – 0.2 nA, carrier gas: IOLAR grade I nitrogen, flow rate 60 mL min^{-1}. The recovery experiment for all isomers of HCH was performed in triplicate and found to be 80–85%. The level of detection and level of quantitation of chlorinated pesticide was 0.01 ppb and 1 ppb respectively.

2.5. Statistical analysis

The experimental characteristics are given as mean ± SE. The variations between the groups for the HCH level and semen parameters were not distributed normally. Therefore, these parameters were analyzed for statistical difference ($p < 0.05$) by Mann–Whitney *U* test. In order to measure the variation between subgroups, Kruskal–Wallis non-parametric ANOVA test was applied. Spearman rank correlation was calculated to measure the association between HCH level and semen quality. In addition, the study population, dietary habits, smoking habits and Y chromosome deletions among fertile and infertile subjects were evaluated.