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

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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 x-HCH and b-HCH in the infertile males. A negative significant association was observed between sperm counts with y-HCH in asthenospermia patients and with y-HCH and total HCH in oligo-asthenospermic patients. Out of 100 males studied, we found 10 patients with Yq deletion in AZFb 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 b-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 a-HCH, b-HCH, y-HCH, d-HCH and e-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
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 oligospermic and azoospermic patients (Vogt et al., 1996). Some candidate genes are recognized in these regions (DFFRY for AZFa, RBBM 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 Makkal 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 × 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 x 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 sulfuric 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; 10LAR 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.
3. Results

3.1. Demographic characteristics

The demographic characteristics of both fertile and infertile groups are shown in Table 1. The mean age of fertile and infertile men was 33.08 and 34.16 years respectively and most of them belong to an urban population. The mean duration of marriage of fertile and infertile men was 8.7 and 9.12 years, respectively. The number of smokers was found to be higher in the fertile than the infertile group. Most of the subjects in both groups were vegetarian.

3.2. Seminal HCH concentration

The average seminal HCH level in different groups is shown in Table 2. The level of seminal HCH analyses were conducted blind in fertile and infertile men. As compared to the fertile group, the concentration of total HCH and its isomers of HCH in semen of fertile men was observed slightly higher than the infertile men. The β-HCH level in semen of fertile men was significantly increased as compared to that in fertile men.

As compared to the subgroups of fertile men with fertile men, the concentration of β-HCH and total HCH were found to be significantly higher in azoospermic patients while the concentration of γ-HCH was significantly higher in asthenospermic patients. The concentration of α-HCH was found higher in oligospermic and asthenospermic patients while the concentration of α-HCH was lower in azoospermic and oligo-asthenospermic patients. The level of seminal β-HCH was increased in azoospermic patients and oligospermic patients and decreased in asthenospermic patients and oligo-asthenospermic patients. The γ-HCH level in semen of oligospermic and asthenospermic patients was found to be higher while the γ-HCH level in semen of oligospermic and oligo-asthenospermic patients was lower. The concentration of δ-HCH in azoospermic patients, oligospermic patients, asthenospermic patients, was found to be lower however, it was higher in oligo-asthenospermic patients. The concentration of total HCH in azoospermic patients, oligospermic patients, asthenospermic patients was found to be higher however, it was lower in oligo-asthenospermic patients.

3.3. Semen quality analysis

The semen quality parameters like viability, motility, sperm counts were observed lower in the infertile group as compared to the fertile group (Table 2). As compared to the fertile men, the mean difference for sperm viability, motility and counts were detected significant (p < 0.05) in all infertile men except sperm motility in oligospermic patients and sperm count in asthenospermic patients. The pH of the semen in all subjects was in the acidic range and no significant differences in colour, odour, viscosity and liquefaction time and volume of semen were observed. None of the samples exhibited pus cells, erythrocytes or agglutinated sperm although a few epithelial cells were noticed rarely.

3.4. Microdeletion analysis

Fig. 1 summarizes the PCR results of patients with microdeletions and the detailed schematic representation of STS based Yq deletions are shown in Fig. 2. In our study, AZFc represents the most frequently deleted region as it showed 10 out of 12 deleted STS markers while AZFa showed only two out of 12 deleted STS markers. No deletion was detected in AZFb region. Out of nine oligospermic patients (Os), Os-2 showed deletion of sY1197 and out of six oligo-asthenic patients (Oa), Oa-2 showed deletion of sY1182. Major deletions were observed in azoospermic patients. Out of 13 azoospermic patients (Az), Az1 showed deletion of sY1161, Az2 showed deletion of sY1258, Az4 showed deletion of sY279, Az5 showed deletion of sY1191, Az6 showed deletion of sY1191, Az7 showed deletion of sY1191, Az12 showed deletion of sY1201 and Az13 showed deletion of sY1180 and sY1258.

3.5. Seminal HCH level versus semen analysis

A correlation of semen quality with seminal HCH level was established in fertile and infertile men, using Spearman rank correlation as shown in Table 3. The α-HCH showed a significant positive correlation with sperm motility (0.323) in fertile men while α-HCH and β-HCH exhibited a significant positive correlation (0.306 and 0.279 respectively) with sperm counts in infertile men. Spearman rank correlation for subgroups of infertile men is shown in Table 4. The γ-HCH showed highly significant negative correlation with sperm count in asthenospermic patients while β-HCH and total HCH had a significant negative correlation (−0.845) with sperm counts in oligo-asthenospermic patients.

3.6. Seminal HCH level versus microdeletion

As shown in Table 2, the microdeletion of Y chromosome and mean difference of β-HCH and total HCH were found significant in azoospermic patients.

3.7. Microdeletion versus semen analysis

The deletion frequency for each category of semen profile is presented in Table 2. Deletions were found in the infertile patients 10/50 (20%), presenting with azoospermic men 8/13 (61.5%), oligospermic men 1/8 (11.1%) and oligo-asthenospermic men 1/6 (16.6%) while no deletions were found in normozoospermic and asthenospermic men.

4. Discussion

In the present study, attempts have been made to evaluate the effects of HCH on reproductive potential of males by measuring the semen quality and PCR based STS analysis of AZF region in fertile and infertile subjects. Due to the lipophilyc and highly persistent nature, HCH accumulate in the living organism and persist in the fat tissues and in fat rich organs for life. Male gonads are highly sensitive target organ for lindane where it damages the...
The results showed a marked increment of HCH level in semen samples of fertile and infertile men from Lucknow, India as compared to the previous study (Pant et al., 2007). The finding of higher seminal concentration of total HCH and its isomers could be explained by continuous exposure to HCH resulting from uncontrolled dumping of hazardous waste of HCH at many places and roadsides in the city and rural areas by the HCH manufacturing plant at Lucknow and unauthorized use by farmers. Prakash et al. (2004) reported the alarmingly high levels of HCH in soil samples from sediments, dumping sites and surrounding areas of lindane manufacturing plant in Lucknow and indicated the high risk of environmental exposure to HCH. The presence of higher concentration of β isomer in semen in our study is due to the inter conversion of γ and α into β isomers in tissues and their persistent nature and slow degradation rate (Nair et al., 1996). The higher concentration of α-HCH, β-HCH and γ-HCH and total HCH in infertile men as compared with fertile men may be responsible for lowering the semen quality and Yq deletions. The apparent small sample size in our study has evoked our interest to undertake analysis of the additional sample to be able to gain a deeper insight into the HCH effect on males.

Several studies have suggested the detrimental effects of HCH on semen quality. Pant et al. (2007) found that β-HCH showed a significant negative correlation with sperm motility while γ-HCH showed a significant negative correlation with sperm count and sperm motility. In our study, the level of α-HCH showed a significantly positive correlation with sperm motility in fertile men and with sperm counts in infertile men while β-HCH showed a significantly positive correlation with sperm counts in the infertile men. The sperm count showed the significant negative correlation with γ-HCH in asthenospermic men while in oligo-asthenospermic, abnormally high levels of HCH in soil samples from sediments, dumping sites and surrounding areas of lindane manufacturing plant in Lucknow and indicated the high risk of environmental exposure to HCH. The presence of higher concentration of β isomer in semen in our study is due to the inter conversion of γ and α into β isomers in tissues and their persistent nature and slow degradation rate (Nair et al., 1996). The higher concentration of α-HCH, β-HCH and γ-HCH and total HCH in infertile men as compared with fertile men may be responsible for lowering the semen quality and Yq deletions. The apparent small sample size in our study has evoked our interest to undertake analysis of the additional sample to be able to gain a deeper insight into the HCH effect on males.

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Correlation between seminal HCH level and semen quality of fertile and infertile men.

Table 3

<table>
<thead>
<tr>
<th>HCH</th>
<th>Fertile</th>
<th>Infertile</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Motility</td>
<td>p Value</td>
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<tr>
<td>α-HCH</td>
<td>0.12</td>
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<tr>
<td>β-HCH</td>
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<tr>
<td>γ-HCH</td>
<td>-0.11</td>
<td>0.44</td>
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<tr>
<td>δ-HCH</td>
<td>-0.01</td>
<td>0.96</td>
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<tr>
<td>Σ-HCH</td>
<td>-0.04</td>
<td>0.79</td>
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</tbody>
</table>

*Correlation is significant at the 0.05 level (2-tailed).

Correlation between seminal HCH level and semen quality of fertile men.

Table 4

<table>
<thead>
<tr>
<th>Infertile men</th>
<th>Motility</th>
<th>p Value</th>
<th>Counts</th>
<th>p Value</th>
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<tr>
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<td>0.95</td>
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<td>0.46</td>
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<td>-0.11</td>
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<td>0.72</td>
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<tr>
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<tr>
<td>β-HCH</td>
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<td>γ-HCH</td>
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<td>Σ-HCH</td>
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*Correlation is significant at the 0.05 level (2-tailed).

*Correlation is significant at the 0.01 level (2-tailed).

lymphocytes taken from three healthy males that increases significantly at 0.1 μL mL⁻¹ technical-grade HCH (6.5% γ-HCH) for 48-h treatment and at 0.05 and 0.1 μL mL⁻¹ for 72-h treatment, suggesting the mild mutagenic activity of this chemical at higher doses in humans (Rupa et al., 1989a,b,c). Several attempts have been made to establish a correlation between HCH exposure, chromosomal aberration and phenotypic changes (Dzwonkowska and Hubner, 1986). However, due to insufficient data, experimental evidence of HCH induced mutations in human still remains highly controversial. Our study demonstrated a correlation between HCH exposure and microdeletion in the AZF region. The microdeletions of Yq represent the aetiological factor in different degrees of hypospermatogenesis (Reijo et al., 1995; Vogt et al., 1996; Foresta et al., 1997, 1998; Simoni et al., 1998). The frequency of Yq deletion was found higher in infertile men. The relatively high frequency of Y deletions pointed out the susceptibility of Y chromosome towards the spontaneous loss of genetic material. The Yq deletions in azospermic patients with a significant mean difference of β-HCH and total HCH may reflect its mutagenic activity. However, the intact germ line DNA of the normospermic males suggested a strong protective mechanism to counter the effects of HCH. Irrespective of the mechanisms involved, the detrimental effects of HCH on Y chromosome was uncovered in the present study. However, the screening of Y chromosome microdeletion in human males with high concentration of seminal HCH would enhance our understanding of the biological consequences of such phenomenon, enabling more focused genotype phenotype correlation. The plausible explanation for this situation involves the susceptibility of male germ line to...
mutagenic changes that could represent a potential risk factor for spermatogenic failure.

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