

Excessive Fluoride Delineating Biochemical Changes in Seminal Plasma: A Case Control Clinical Study

DUSHYANT SINGH CHAUHAN

SANDEEP TRIPATHI¹

VIVEK PRATAP SINGH

National Referral Centre for Fluoride Poisoning in India

Nims University, Jaipur

Institute of Advanced Sciences and Technology

Nims University, Jaipur

India

SURABHI TOMAR

SHOBHA TOMAR

National Referral Centre for Fluoride Poisoning in India

Nims University, Jaipur

Nims Fertility and Research Centre

Nims Medical College & Hospital, Nims University, Jaipur

India

MUKESH TIWARI

National Referral Centre for Fluoride Poisoning in India

Nims University, Jaipur

Department of Orthopaedics, Nims Medical College & Hospital

Nims University, Jaipur

India

ABBAS ALI MAHDI

Department of Biochemistry

King George's Medical University, Lucknow

India

Abstract:

Fluorosis has become an endemic problem in India and worldwide. Significant interest has occurred on potential decline in the semen quality due to contamination of fluoride in drinking water. The

¹ Corresponding author: Assistant Professor of Biotechnology, Institute of Advanced Sciences & Technology, Head (R & D) National Referral Centre for Fluoride Poisoning in India, Nims University, Shobha Nagar, Jaipur-303121 India, E-mail: sandeeptripathiphd@gmail.com

aim of the present study was to investigate the possible impact of fluoride exposure on the semen quality. In the present study, 150 subjects (age 25-40) were recruited from the high fluoride region of Rajasthan, India, where fluoride content in ground water was more than 2.0 ppm. The age matched controls were selected from the area where fluoride level was less than 1.5 ppm. The total protein, fructose, lipid, phospholipids and cholesterol levels were estimated in seminal plasma and correlates with the semen volume, liquefaction time, viability and motility followed by the estimation of fluoride concentration in serum and urine. A significant ($p < 0.05$) reduction was observed in fructose, protein, lipids and phospholipids while the concentration of cholesterol was raised in subjects as compared to controls. Diminished semen quality in term of Volume, Liquefaction time, Viability and motility were noticed along with the elevated levels of fluoride in serum and urine. On the basis of results it may conclude that high exposure of fluoride through drinking water may be associated with reduced semen quality in population of endemic fluorosis areas.

Key words: Fluoride; NRCFPI, Semen, Biochemical Changes

Introduction

The environmental contamination has been suggested to play a role in adverse reproductive health effects including diminished semen quality (Sun et al., 2011; Pant et al., 2004). It has been suggested that many environmental factors including chemical substances, smoking and other pollutants present in air, water, and soil may be responsible for reproductive physiology (Lazaridis et al., 2008). Among these chemical, fluoride (F) is abundantly found in the ground drinking water in worldwide and very little amount is required to humans (Rango et al., 2014). High fluoride concentration in soil & water due to this accumulation of high fluoride concentration in drinking water may lead to health problem in humans. Fluoride poisoning from long-term exposure to high levels of fluoride is a serious health

problem in many parts of the world where drinking water contains more than 1.5 ppm of fluoride (WHO 1984).

Endemic fluorosis is prevalent in India since 1937 (Shortt et al., 1937). It has been estimated that the total population consuming drinking water containing elevated levels of fluoride is over 66 million (FRRDF, 1999). The available data suggest that 15 States in India are endemic for fluorosis (fluoride level in drinking water >1.5 mg/l), and about 62 million people in India suffer from dental, skeletal and non-skeletal fluorosis. Rajasthan have high fluoride (up to 24 ppm) in their drinking/ground water sources and about 11 million of the populations are at risk (ICMR & RMRCT, 2005; Yadav et al 2003). Moreover in state of Rajasthan, people of 22 districts (out of 33 districts) are presently consuming Fluoride (Samal & Naik, 1988) greater than permissible limit.

High concentration of fluoride influences the sperm count and quality and damage testis, epididymis and prostate structure so as to influence male reproductive ability (Prystupa, 2011). Various studies indicated that fluoride reflects certain reproductive damage and endocrine-disrupting effects through influencing multiple hormone levels of the hypothalamic-pituitary-gonadal axis in animal and humans (Pengfei et al., 2010; Chauhan et al., 2013). There are various researches have been conducted to explore the relationship between fluoride ingestion and reproductive structure in animal models (Jiang et al., 2005; Xu et al., 2010). The reproductive toxic effects are abnormal spermatozoa, loss of spermatogenesis in rats, decreased sperm quality and quantity. But exact mechanism of the toxicity is not well understood in the clinical samples which required to be scientifically investigated. To fill these scientific lacunae, the present study was undertaken. Keeping in view the paucity of information in relation to high fluoride exposure in population residing in endemic areas and its impact on reproductive system, the present study was undertaken.

Method

In the present study, 150 male fluorosis cases were selected from the area where fluoride in water was more than 2.0 ppm. The age matched controls were selected from the area where fluoride content in water was less than 1.5 ppm. The controls (non fluorotic) and subject (fluorotic cases) were confirmed after the performance of physical test and Dean's index (Susheela et al., 1993; Dean 1934), and fluoride content in the serum of control and subjects. The study was approved by the Institutional human ethical committee. A written consent of each subject was taken after explaining the aims and objectives of the study and its benefit to individual and society.

A detailed medical history and andrological examination was performed for all studied cases. Subjects currently on any medication or antioxidant supplementation were not included. Also, patients with varicocele, leucospermia, those were suffering from any acute infection, smokers and alcoholic men were excluded from the study. The subjects were intervened using personal interview and detailed information of the subjects were recorded on the pre-designed performa which includes age, BMI, socio-economic status, educational level, smoking, alcohol, marital status, number of children, drug addiction and contraception.

Water Analysis

The drinking water samples were collected from each subjects and controls in clean polyethylene bottles in all seasons for a period of 2011-2013. Temperature was determined in the field. The pH (pH meter), turbidity (Nephelometer) and total dissolved solids (by evaporation method) were determined. Total alkalinity, dissolved oxygen, bicarbonates, hardness, free CO₂, magnesium, calcium, nitrates, chloride, fluoride, were analyzed by volumetric titration method. Sodium and

potassium were measured by digital flame photometer. The concentration of fluoride in drinking water was analysed using an ion-specific electrode Fluoride Electrode Instrument (Orion, Singapore). Following procedures were carried out.

Fluoride estimation

After clinical examination of subjects and controls, 3.0 ml of blood sample was drawn under complete aseptic condition in sample vial and was allowed to clot at room temperature for serum separation. The first urine at morning of each individual was collected. Furthermore, fluoride concentrations were measured in both serum and urine using specific fluoride electrode (Thermo Fischer, Singapore).

Semen Collection

Semen samples were collected from the subjects and controls in a clean, dry, sterilized, wide mouth, well stopper glass vial by masturbation after 2–5 days of abstinence. After liquefaction, semen samples were centrifuged at $1200 \times g$ in cold (4°C) for 20 min for the separation of seminal plasma. The supernatant (seminal plasma) was centrifuged again at $10\,000 \times g$ in cold (4°C) for 30 min to eliminate all possible contaminating cells and stored at -20°C until analyzed.

Biochemical Estimations

The protein content was measured by the standard method of Lowry et al, (1952) using bovine serum albumin (BSA) as standard. The concentration of protein was expressed as mg protein/ml seminal plasma. The level of fructose was estimated by indole method (Karvonen and Malm, 1955) using zinc sulphate. The concentration of fructose was expressed as mg fructose/ml in seminal plasma. The total lipids were estimated according to the method of Woodman and Price (1972). Lipids from seminal plasma were extracted in chloroform: methanol mixture (2:1, v/v) by the method of Folch et al. (1957). The

results were expressed in mg/ml of lipids in seminal plasma. Total phospholipids were calculated by estimating phosphorus following the method of Fiske and Subbarao (1925) as modified by Marinetti (1962). Organic phosphorus of phospholipids was converted to inorganic phosphorus by digesting the lipid with perchloric acid. The results were expressed in mg/ml of phosphorous. The total cholesterol was estimated according to the method of Zlatis et al., (1954). The cholesterol polymerized in the presence of acetic acid and reacts with ferric chloride to form a violet colour complex which is measured by spectrophotometer at 570 nm. Standard curve of cholesterol was used to calculate the amounts of cholesterol in the seminal plasma and it was expressed as mg/ml of cholesterol in seminal plasma.

Statistical Analysis

The data were summarized as Mean \pm SD. Groups were compared together by one way analysis of variance followed by Student Newman-Keuls post hoc test. The acceptance level of significance was $p < 0.05$. InStat (version 3) was used for analysis of data.

Results

Various parameters like temperature, pH, turbidity, total dissolved solids, total alkalinity, D.O., bicarbonates, total hardness, magnesium, calcium, nitrates, chloride and fluoride were investigated in drinking water of control and subjects. All physical parameter compared with the Bureau of Indian standards (BIS) in table -1. The range of pH was within the limit and the total hardness (CaCO_3 mg/L) was found to be insignificant change in control and subjects. Trace minerals namely, Calcium, Chloride, Manganese, Nitrate, Iron, Sulphate, Lead, Arsenic, Aluminium, Mercury, Chromium,

Cadmium, Selenium and Zinc (mg/L) present in the drinking water of control and subjects as per the BIS standard. There were insignificant ($p>0.05$) change between groups. There was no nonstandard turbidity in the water sample.

The concentration of fluoride in water was found to be as markedly ($p<0.001$) higher in the drinking water of each subject when compared with the controls. The recommended fluoride concentration in drinking water is 1.5ppm (WHO).

There was statistically insignificant ($p>0.05$) difference between the subjects and controls regarding their age, BMI, literacy, socioeconomic status. In this study all the subjects and controls were married and literate, moreover they are not drug addicts and male contraceptive users. While, smoking and alcoholic habit were found occasionally and insignificant ($p>0.05$) difference found between control and subjects.

The concentration of fluoride in urine and serum was also estimated in control and subjects. The fluoride concentrations in urine are presented in figure -1. There was significantly ($p<0.001$) increased in subjects when compared with controls. Moreover concentration of fluoride in serum is presented was also found to be markedly ($p<0.001$) increased in subjects as compared with the controls.

The semen profiles were carried out in control and subjects and data are presented in table-3. The semen volume was found to be significantly ($p<0.001$) reduced by 20% and liquefaction time was increased by 67%, in the subject as compared to the controls respectively. The viability and motility were reduced by 15% and 23% in subjects when compared with the controls respectively.

The biochemical profiles namely fructose, protein, lipid, phospholipids and cholesterol were investigated in the seminal plasma of control and subjects groups. The data are presented in table-4. The concentration of fructose in seminal plasma of subjects was found to be significantly ($p<0.001$) decreased by 31% in subjects when compared with controls respectively.

Protein in seminal plasma of subjects was found to be reduced by 12% ($p < 0.001$) as compared with the controls. Lipid in seminal plasma of subjects was found to be markedly reduced by 13% ($p < 0.001$) in subjects when compared with controls. The phospholipids in seminal plasma was significantly ($p < 0.001$) reduced by 18% in subjects when compared with the controls. While, the level of cholesterol in seminal plasma was found to be markedly ($p < 0.001$) elevated by 14% in subjects as compared with the controls.

Discussion

Fluorosis is a major public health concern in India and worldwide due to the excessive consumption of fluoride through drinking water. In India, Rajasthan seems to be threatening area of fluoride toxicity in drinking water. Most of the fluorosis cases in Rajasthan are caused by the high concentration of fluoride in drinking water. Various fluoride affected regions were selected with the help of National Referral Centre for Fluoride Poisoning in India, Nims University, Jaipur India and the water samples have been taken for the analysis of water fluoride content. Water samples from different bore wells of selected regions showed a maximum range of 2.6 to 14.7 ppm by fluoride electrode (Table -1). Almost all the selected area is higher fluoride than the permissible limit of 1.5 ppm (WHO, 1984). Analysis of the water samples showed the fluoride content in abnormal range both in urine and serum (figure-1 and 2), while accepted average normal serum fluoride value is 0.15 ppm as estimated by Singer and Armstrong (1977).

A great number of substances have been found in seminal plasma but so far it has not been possible to provide evidence of clinical significance for all of them. In the light of most current knowledge, fructose occupies the most important place in biochemical investigation (Lewis-Jones et al., 1996). The protein spectrum in the seminal plasma is formed mainly

by the seminal vesicular fluid proteins and to a lesser extent, by the proteins in the fluids of the cauda epididymides and prostate (Dostal and Vaselsky 1972). Among many components of seminal plasma, proteins and peptides play a specific role in regulation of the fertilization process, particularly through their ability to bind various types of ligands. Although many studies demonstrated that the seminal plasma proteins may function to stabilize the sperm against premature capacitation and spontaneous acrosome reaction (De Lamirande et al., 1997). In the present study, reduced protein concentration was observed and it may be due to protein oxidation or caused lesions in reproductive organs by fluoride. Mahdi et al., (2011) also provide evidence that reduced concentration of protein may be responsible for infertility.

Fructose acts as a donor of energy to the spermatozoa, which break it down selectively and convert it into energy (Schoenfeld et al., 1979). Fructose is reported to play important roles in sperm motility and concentration, particularly with regard to energy metabolism. Fructose is closely involved in certain aspects of energy metabolism, through glucose utilization. Fructose is one of the major energy yielding nutritive substrates present in human seminal fluid (Sanchez-Partida et al, 1999). In the present study, reduced fructose level in seminal plasma suggests that impaired energy balance leads to reduced motility and viability (Aumuller and Riva 1992). Therefore, sugar composition of seminal plasma may correlate with fertility, mainly because of its importance for sperm energy production (Garner et al, 2001). Various lipid molecules play an important role in structural and functional basis of spermatozoa. Spermatozoa membranes that derive from spermatogonia and spermatocytes are considerably modified through changes in their cholesterol and phospholipid composition during spermatogenesis, sperm maturation and capacitation (Cross, 1998; Jones, 1998; Flesch and Gadella, 2000; Flesch et al., 2001).

Lipid is a key feature in the function of spermatozoa. Lipid profiles consist of cholesterol, phospholipids, diglycerides, triglycerides, and wax esters. The possible source of lipid in seminal plasma could be the epididymides, as well as spermatozoa (Pickett and Komarek, 1966). Seminal lipids—specifically phospholipids and cholesterol—have special relevance in the structure and function of the spermatozoa (Cross, 1998) and might play significant roles in the sperm structure, metabolism, sperm capacitation, and fertilization of female gametes (Hafez, 1987). In this study, reduced concentration of lipids and phospholipids and increased level of cholesterol were observed in seminal plasma of fluorosis patients (Table-4). It is suggestive that, fluoride may potentiate the generation free radical and oxidative damage. Our previous study, demonstrated that fluoride increases lipid peroxidation through the generation of reacting oxygen species (Chauhan et al., 2013a). Sperm plasma membrane composed of polyunsaturated fatty acids and they are highly susceptible for lipid peroxidation and protein oxidation (Chauhan et al., 2013b).

The increased level of cholesterol in subjects is an indicator of extracellular cholesterol which may be responsible for reduced motility and viability. Moreover, it may also associate with delayed liquefaction time. Beer-Ljubic (2009) also demonstrated that increased cholesterol is predictive marker for quality semen evaluation. Moreover, Deterioration in cholesterol concentration in seminal plasma was positively correlated with sperm motility, concentration and total number of sperm by Jacyno et al., 2009. Furthermore, it is proven in this study that fluoride caused significant deterioration in semen quality in the fluoride exposed populations (Table-3).

In the present study exhibited reduced semen volume, viability and motility and increased liquefaction time in fluorotic patients. Sperm motility becomes critical at the time of fertilization and it is one of the biological characteristics of the

spermatozoa. The poor motility is called asthenozoospermia which is associated with reduced viability of spermatozoa. It is suggestive that fluoride interferes with the sperm physiology either by direct contamination of fluoride in testicular organelles or may be through alteration in biochemical changes. The decline in sperm viability and motility as in results are concomitant with the finding of Ghosh et al., (2002). They showed fluoride induced testicular toxicity in rats.

Conclusion

From the results obtained in this research work we can conclude that reduced semen quality may be correlate with altered biochemical changes in seminal plasma induced by fluoride toxicity. As we found reduced motility and viability in fluorosis subject it may be due to impaired energy balance. Possibly this phenomenon is caused by the relation between the proportion of lipids, fructose and protein and their metabolism. Since, quality of Sperm is an important index of impaired reproductive function and biochemical profiles are the indicators of the quality of sperm.

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Table-1. Physico - Chemical Characteristics of drinking water of control and subject

Parameters	BIS	Control	Subject
pH	6.5 - 8.5	7.1 ± 0.5	7.2 ± 0.4
Total Hardness (CaCO ₃ mg/L)	300 - 600	400 ± 45.2	560 ± 43.5
Calcium (mg/L)	75-200	115 ± 14.4	160 ± 11.5
Chloride (mg/L)	250 - 1000	460 ± 100.4	550 ± 69.8
TDS (mg/L)	500 - 2000	1150 ± 201.7	1060 ± 187.4
Fluoride (mg/L)	1.0-1.5	0.9 ± 0.2	5.6 ± 1.9*
Manganese (mg/L)	0.10 -0.30	0.16 ± 0.07	0.24 ± 0.04
Nitrate (mg/L)	45 - 100	56.8 ± 3.8	68.8 ± 8.1
Iron (mg/L)	0.3 - 1.0	0.5 ± 0.1	0.55 ± 1.0
Sulphate (mg/L)	200 - 400	315± 23	325 ± 34
Lead (mg/L)	0.05	0.03 ± 0.01	0.02 ± 0.01
Arsenic (mg/L)	0.01	0.006 ± 0.002	0.004 ± 0.005
Aluminium (mg/L)	0.03	0.02 ± 0.005	0.02 ± 0.005
Mercury (mg/L)	0.001	0.0003 ± 0.0001	0.0004±0.0001
Chromium (mg/L)	0.05	0.04 ± 0.005	0.04 ± 0.006
Cadmium (mg/L)	0.01	0.008 ± 0.001	0.006 ± 0.001
Selenium (mg/L)	0.01	0.008 ± 0.001	0.008 ± 0.001
Zinc (mg/L)	5.0	2.6 ± 1.1	3.4 ± 1.1

Data are presented as Mean ± SD

Table-2. Demographic data of control and subjects.

	Control (N=150)	Subject (N=150)
Age	32.5 ± 6.8	33.4 ± 5.2
BMI	21.3 ± 1.1	22.1 ± 1.0
Socio-economic status	Lower (100%)	Lower (100%)
Married	100%	100%
Literacy (H.Sc.)	100 %	100 %
Smokers	10%	9%
Alcoholic (Occasionally)	4.3%	3.3%
Drug addicted	Nil	Nil
Male Contraceptive	Nil	Nil

Data are presented as Mean ± SD

Table-3. The semen physiology of in control and subjects.

Seminal Profile	Control (150)	Exposed (150)	P Value
Semen Volume (ml)	3.88 ± 0.5	3.11 ± 0.7	P<0.01
Liquefaction time (min)	14.8 ± 2.4	24.7 ± 7.7	P<0.01
Viability (%)	65.2 ± 16.2	55.2 ± 20.1	P<0.05
Motility (%)	70.8 ± 17.3	54.5 ± 14.4	P<0.001

The semen parameters of subjects and control are expressed as mean ± SD for control and subjects. Superscripts relate significant (minimum, p< 0.05; One way ANOVA followed by students-Newman Keules test.

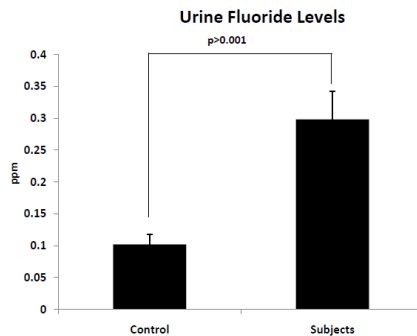
Table-4. The Biochemical Parameters in seminal plasma of control

	Control	Subjects	P- value
Fructose (mg/ml)	3.26 ± 1.1	2.24 ± 1.4	P<0.001
Protein (mg/ml)	82.10 ± 14.0	72.60 ± 13.0	P<0.01
Lipid (mg/ml)	36.09 ± 5.2	31.33 ± 7.5	P<0.05
Phospholipids (mg/ml)	11.01 ± 4.4	09.06 ± 4.4	P<0.05
Cholesterol (mg/dl)	42.00 ± 12.0	48.30 ± 13.0	P<0.01

and subjects.

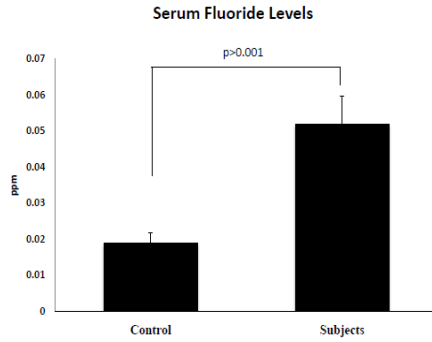
The concentration of Fructose (mg/ml), Protein (mg/ml), Lipid (mg/ml), Phospholipids (mg/ml) and Cholesterol (mg/dl) are expressed as Mean ± SD in control and subjects. Superscripts relate significant (minimum, p< 0.05; One way ANOVA followed by students-Newman Keules test.

Figure-1



The concentration of urine fluoride is expressed as Mean ± SD in control and subjects. Superscripts relate significant (minimum, p< 0.05; One way ANOVA followed by students-Newman Keules test.

Figure-2



The concentration of serum fluoride is expressed as Mean \pm SD in control and subjects. Superscripts relate significant (minimum, $p < 0.05$; One way ANOVA followed by students-Newman Keules test).