THE ALTERATIONS IN GROUND SUBSTANCES AND COLLAGEN METABOLISM IN CASES OF CHRONIC FLUORIDE INTOXICATION BEFORE AND AFTER SUPPLEMENTATION WITH CALCIUM, VITAMIN C(ASCORBIC ACID) AND VITAMIND

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ABSTRACT

Background: The aim of present study was to find out the effects of excess of fluoride on ground substance and collagen metabolism and to assess the effects of supplementation of vitamin C, D and calcium on fluoride intoxication. **Material and Method:** The study was conducted on 100 children of age group 5 to 12 years selected from three fluoride belts of Jaipur district (25 children from each area consuming water with 2.4, 5.6, & 13.6 mg/l of fluoride) compared with 25 controls, consuming water with normal fluoride levels. Drinking water fluoride, serum and urinary fluoride levels were measured by ion selective electrode method. Urinary hydoxyproline, Serum ascorbic acid, Serum calcium, Urinary calcium, Serum sialic acid, Serum hexosamine, Serum inorganic phosphate were estimated by spectrophotometic method. **Result:** The study indicated an increase in levels of urinary hydoxyproline and decrease in ascorbic acid levels with increase in drinking water fluoride, similarly levels of serum sialic acid and serum hexosamine decreased with increase in fluoride concentration and an increase in urinary calcium was also observed but serum calcium levels and inorganic phosphate levels were found to be normal. Treatment with calcium, Vitamin C and Vitamin D showed reduction in serum fluoride, increase in urinary fluoride concentration and decease in urinary calcium levels and decrease in urinary hydroxyproline levels. **Conclusion:** Fluoride intoxication due to drinking water can result in abnormalities in ground substance metabolism which can be resolved by proper supplementation with calcium, vitamin C and D.

Key words: Chronic fluoride intoxication, hydroxyproline, collagen, ground substance, ascorbic acid, vitamin D

INTRODUCTION:

Fluoride is an essential trace element in human metabolism. WHO permits 1.5 ppm as a safe limit for human consumption (1) Fluorosis a slow debilitating disease results due to excess quantity of fluorides in drinking water. Rajasthan is one of the most affected areas. A total of 11909 villages and 11388 other habitation (24.79% of total) are having fluoride levels in their ground water in concentration of over 1.5 ppm (2).

Fluoride toxicity leads to number of biological effects i.e. effects on bone, teeth, kidneys,

thyroid, haematological functions and growth in general and it also increases aging process. Fluoride exposure disturbs synthesis of collagen and leads to its breakdown which is manifested as increase in excretion of hydroxyproline in urine. Ascorbic acid which is involved in synthesis of collagen was also found to be low in patients suffering from Fluorosis. (3, 4)

Many workers have reported association of disturbed collagen metabolism and low levels of serum ascorbic acid (4).Fluoride toxicity also disturbs the metabolism of amorphous ground substance manifested as altered levels of sialic acid and hexosamine in blood and serum. Fluoride toxicity disturbs calcium homeostasis with concomitant changes in parathyroid hormone which results in causing important changes in bones and other calcified tissues.

Combination of calcium, vitamin D and ascorbic acid supplementation could reverse Fluorosis at least in children's. (5, 6)

The present study was therefore planned to correlate biochemical parameters with fluoride in drinking water and find out relationship between ascorbic acid and collagen metabolism.To find out magnitude of changes in ground substance.To find out effects of dietary supplementation of vitamin C and calcium on parameters under study.

MATERIAL AND METHODS

The study was cross sectional which included three villages of Jaipur district where the concentration of fluoride in drinking water was above normal limit of 1.5 ppm.

Children in the age group of 5 to 12 years were selected randomly from these three areas namely:

- Jaipur city (control group) 1.2 ppm Group I
- Ramsagar ki dhani 2.4 ppm Group II
- Shivdaspura 5.6 ppm Group III
- Raipuria 13.6 ppm Group IV

The criteria for the selection were different levels of fluoride above 1.5 ppm in drinking water in these villages. The study was conducted on 100 children. 25 children were selected from each of three affected areas presenting with dental fluorosis and 25 were selected from control (area having<1.5 ppm fluoride).

An informed consent was obtained from parents of all children after explaining the purpose of the study. Clinical examination was done by paediatrician for the presence of any chronic disease. 3 ml of blood was drawn in the morning under aseptic precautions at the start of study i.e. before beginning of supplementation and at the end of six month of supplementation.

Serum and urinary fluoride were estimated by ion selective electrode method (7), Urinary hydroxyproline was assessed by method given by N-Raghumanulu et al (8), serum ascorbic acid by method given by Natelson (9), serum calcium and Urinary calcium by OCPC method (10), serum sialic acid by Seibert and Seibert method (11), serum hexosamine by Elson and Morgan method (12) and serum inorganic phosphate and alkaline phosphatase detected by Varley (13,14).

Supplementation was provided to all children included in study consisting of 500 mg of ascorbic acid (Vitamin C) thrice daily and 1 gm of elemental calcium given in form of calcium carbonate packaged in gelatine capsules in two divided doses morning and evening immediately following meals.

Vitamin D was given in doses of 6000 IU biweekly.Blood and urine samples were

collected at the end of six month of supplementation for analysis and the same parameters were estimated.

The results were statistically analysed using paired Students' t test and level of significance was $p< 0.05$.

RESULT

Serum fluoride levels were observed to be high as compared to normal in all test groups ranging from 0.16-1.03 ppm before and after supplementation and levels come down significantly after supplementation in group II, III and IV (Table-1).

Mean serum calcium levels were found to be in normal range in all groups including control group before and after supplementation. The range observed in pre supplementation group being 9.64±0.38, 9.16±1.29, 9.5±1.05 and 10.6±1.02 mg% in I, II, III, and IV respectively. The small changes in levels of serum calcium after supplementation reflect a readjustment in levels due to supplementation therapy (Table-1).

Serum inorganic phosphorus values were found to be well within normal range. After supplementation a significant change was observed in control, group II and IV (Table-2).

Mean serum alkaline phosphatase values in pre and post treatment groups are shown in table-3. The mean values were high in all pre treatment groups. After supplementation a significant reduction was observed in group II, III and IV ($p< 0.001$) but in control group change observed was not significant.

Sialic acid levels were found to be very much below the reported normal values in all the groups. After supplementation a significant improvement was observed and was found to be significant in all groups with $p<0.001$(Table-4).

Serum hexosamine were found to be variable in different groups being normal in group II and III and higher than normal in group IV. After supplementation the levels came down significantly in group I (Table-5).

Serum Ascorbic acid levels were also observed in normal limits ranging from 0.58-1.23 mg%. After supplementation the levels increased significantly (Table-6).

Urinary Fluoride levels are considered as a parameter to assess the magnitude of fluoride intake. In present study the Urinary Fluoride excretion was found to be significantly high in test group II, III and IV while in group I excretion of fluoride was within normal range. After supplementation a significant increase in fluoride excretion was observed in all test groups (Table-7).

Urinary Hydroxyproline a unique amino acid found only in collagen tissues is an important parameter to assess metabolism of collagen tissue. The urinary excretion of hydroxyproline was found to be significantly high in all test groups in pre-supplementation category and levels were found to correlate positively with drinking water fluoride concentration. After supplementation the hydroxyproline excretion in urine decreased significantly (Table-8).

Normal Urinary Calcium ranges from 10-24mg%. In present study urinary calcium excretion was found to be higher in group II, III and IV and after supplementation levels came down except in group IV where an increase was observed (Table-9).

Observation tables

Table 1: Comparison of serum fluoride levels with serum calcium levels before and after supplementation

	Serum Fluoride(ppm)		Serum Calcium(mg/dl)	
Groups	Pre supplementation	Post supplementation	Pre supplementation	Post supplementation
I	0.16±0.05	0.14+0.05 NS	9.64+0.38	9.66+0.3 NS
II	0.70±0.10	0.60+0.10 *	9.16+1.29	9.88+1.29 *
III	0.60±0.15	0.38+0.15 *	9.50+1.05	8.53+1.05 *
IV	1.03±0.13	0.81+0.13 *	10.6+1.02	9.37+1.02 *

All values are mean+SD, *p<0.001; NS not significant

Table 2: Comparison of serum fluoride levels with serum inorganic phosphorous levels before and after supplementation

	Serum Fluoride (ppm)		S
Groups	Pre supplementation	Post supplementation	P
I	0.16±0.05	0.14±0.05 NS	5.
II	0.70±0.10	0.6±0.1*	3.
III	0.60±0.15	0.38±0.15*	5.
IV	1.03±0.13	0.81±0.13*	4.

All values are mean±SD

*p<0.001; **p<0.05; NS not significant

Table 3: Comparison of serum fluoride levels with serum alkaline phosphatase levels before and after supplementation

	Serum Fluoride(ppm)		Serum alkaline phosphatase(KAunits)	
Groups	Pre supplementation	Post supplementation	Pre supplementation	Post supplementation
I	0.16+0.05	0.14+0.05 NS	24.50+4.02	25.60+4.02NS
II	0.70+0.10	0.60+0.10 *	27.6+5.90	20.55+5.90*
III	0.60+0.15	0.38+0.15 *	40.18+10.95	28.94+10.95*
IV	1.03+0.13	0.81+0.13 *	26.50+3.22	21.32+3.22*

All values are mean+SD

*p<0.001; NS not significant

Table 4: Comparison of serum fluoride levels with serum sialic acid levels before and after supplementation

	Serum Fluoride(ppm)		Serum sialic acid(mg/dl)	
Groups	Pre supplementation	Post supplementation	Pre supplementation	Post supplementation
I	0.16±0.05	0.14±0.05 NS	12.30±20	13.2±20**
II	0.70±0.10	0.60±0.10 *	7.27±1.57	9.86±1.57 *
III	0.60±0.15	0.38±0.15 *	4.73±1.36	5.58±1.36 *
IV	1.03±0.13	0.81±0.13 *	4.06±3.19	6.10±3.19 *

All values are mean±SD

*p<0.001; **p<0.05; NS not significant

Table 5: Comparison of serum fluoride levels with serum hexosamine levels before and after supplementation

	Serum Fluoride(ppm)		Serum hexosamine(mg/dl)	
Groups	Pre supplementation	Post supplementation	Pre supplementation	Post supplementation
I	0.16±0.05	0.14±0.05 NS	37.5±3.99	40.20±3.99*
II	0.70±0.10	0.60±0.10 *	111.00±45.50	97.50±45.50 NS
III	0.60±0.15	0.38±0.15 *	96.55±30.35	105.00±30.35 NS
IV	1.03±0.13	0.81±0.13 *	192.00±54.30	113.50±54.30*

All values are mean±SD

*p<0.001; NS not significant

Table 6: Comparison of serum fluoride levels with serum ascorbic acid levels before and after supplementation

	Serum Fluoride(ppm)		Serum ascorbic acid(mg/dl)	
Groups	Pre supplementation	Post supplementation	Pre supplementation	Post supplementation
I	0.16±0.05	0.14±0.05 NS	1.08±0.14	1.07±0.14 NS
II	0.70±0.10	0.60±0.10 *	1.23±0.33	1.46±0.33 *
III	0.60±0.15	0.38±0.15 *	1.06±0.63	1.36±0.63 **
IV	1.03±0.13	0.81±0.13 *	0.58±0.45	0.82±0.45 **

All values are mean±SD

*p<0.001; **p<0.05; NS not significant

Table 7: Comparison of serum fluoride levels with urinary fluoride levels before and after supplementation

	Serum Fluoride(ppm)		Urinary fluoride(	
Groups	Pre supplementation	Post supplementation	Pre supplementation	Post supplementation
I	0.16±0.05	0.14±0.05 NS	0.48±0.11	0.45±0.11 NS
II	0.70±0.10	0.60±0.10 *	9.95±0.78	10.90±0.78*
III	0.60±0.15	0.38±0.15 *	15.00±0.72	17.06±0.72*
IV	1.03±0.13	0.81±0.13 *	13.30±1.83	14.64±1.83*

All values are mean±SD

*p<0.001; NS not significant

Table 8: Comparison of serum fluoride levels with urinary hydroxyproline levels before and after supplementation

	Serum Fluoride(ppm)		Urinary hydroxyproline (mg/24hrs.)	
Groups	Pre supplementation	Post supplementation	Pre supplementation	Post supplementation
I	0.16±0.05	0.14±0.05 NS	51.80±7.45	52.40±7.45 NS
II	0.70±0.10	0.60±0.10 *	120.00±27.80	58.70±27.80*
III	0.60±0.15	0.38±0.15	149.80±33	41.50±33.

		*	.29	29*
IV	1.03±0.13	0.81±0.13 *	266.00±26 .50	61.00±26. 50*

All values are mean±SD

*p<0.001; NS not significant

Table 9: Comparison of serum fluoride levels with urinary calcium levels before and after supplementation

	Serum Fluoride(ppm)		**Urinary calcium (mg/24hrs.)**	
Groups	Pre supplementation	Post supplementation	Pre supplementation	Post supplementation
I	0.16±0.05	0.14±0.05 NS	20.10+4.94	21.00+4.94 NS
II	0.70±0.10	0.60±0.10 *	94.10+43.00	72.50+43.00
III	0.60±0.15	0.38±0.15 *	107.60+37.53	77.40+37.53
IV	1.03±0.13	0.81±0.13 *	105.00+28.30	130.00+28.30

All values are mean±SD

*p<0.001; NS not significant

DISCUSSION

Hydroxyproline is unique amino acid found only in collagen tissue of body and its excretion in urine is a marker to follow changes in collagen metabolism under experimental conditions and in various diseased states (15). Normally excretion ranges between 9-61 mg%. In present study the urinary hydroxyproline levels in all pre supplementation test groups ranges between 73.12 mg% to 266 mg% i.e. higher than normal and levels decreased significantly after supplementation and come down to normal but changes in control group were not significant. These findings suggest that there is increased degradation of collagen during fluoride toxicity causing increased urinary hydroxyproline levels. These levels decreased after supplementation due to decrease in collagen breakdown with increased synthesis of new collagen.

Ascorbic acid influences the metabolism of ground substance specially the collagen (Normal levels 0.4-1.5 mg %). Exposure to fluoride causes reduction in ascorbic acid content (3, 4) in children consuming water with fluoride content up to 13 ppm and this reduction does not allow the conversion of proline and lysine bound to peptide linkage to hydroxyproline (16, 17) and so synthesis of collagen is affected and more over hydroxyproline functions to stabilize the collagen triple helix agent digestion by proteases (18, 19).

In present study ascorbic acid in serum in pre treatment category was found to be within normal range but various studies suggest a decrease in levels of serum and leukocyte ascorbic acid in children consuming water containing fluoride up to 13 ppm.

Serum calcium also plays important role, and in this study increase in fluoride content decreased serum calcium levels in all groups under the continuous exposure to excess fluoride in drinking water. The reason is that a slight decrease in serum calcium brings about an immediate release of PTH which maintains serum calcium within normal range. The Fluorosis affects the functions of PTH by affecting serum calcium (20). Normal levels of serum calcium were observed along with increase in PTH in children consuming high concentration of fluoride in drinking water but this was corrected after supplementation of calcium and vitamin D (21).

Assessing urinary calcium in pre and post supplementation group supports the above facts. In present study the urinary calcium levels were found to be within normal range in group I both before and after supplementation but in test group II,III,IV urinary calcium levels were much higher than normal and levels came down significantly after supplementation except in group IV. The findings suggest that increase in urinary calcium excretion before supplementation may be due to increased removal of calcium from bones due to greater release of PTH in wake of hypocalcaemia caused by increased consumption of fluoride.

After supplementation with calcium the release of PTH is decreased, so more calcium is available for deposition in bones causing decreased calcium excretion after treatment. Serum inorganic phosphorus was also assessed as there is reciprocal change in level of phosphorus as compared to calcium. In the wake of strict parathyroid control the levels of both calcium and phosphorus have been observed within normal limits. Grooten et al (22) also reported that dietary fluoride does not influence urinary or plasma levels of calcium and phosphorus.

Serum fluoride levels were found to be elevated in group II, III, IV as compared to control group in pre supplementation group. After supplementation the levels came down significantly in all test groups but changes in control group was non- significant. After supplementation of calcium, vitamin D and C significant changes were observed in test group due to increase in renal clearance secondary to decreased absorption of fluoride from GIT. This has effect on urinary fluoride excretion which is considered to be best indices of fluoride intake (23).

A significant increase in urinary fluoride excretion was observed in all test groups after supplementation indicating a definitive role of dietary supplementation of vitamin C, D and calcium in reducing fluoride accumulation in body.

A significant positive correlation has been observed between serum urinary fluoride excretion in control and group IV (r value=+0.684 & 0.47.) Gupta et al (4) and other workers have reported increased fluoride excretion following increased ingestion of fluoride. Estimation of serum alkaline phosphatase was also done and the levels of alkaline phosphatase have been observed to be on higher side in all the studied groups indicating osteoblastic bone activity in subjects indicating laying down of new bones. Rajyalaxmi et al (4, 24, and 25), Ming HoYu et al 1988 and Gupta SK 1999 observed high enzyme activity in fluorosis.

Serum hexosamine is also an important constituent of ground substance. Its levels in serum ranges between 75-120 mg %. In present study it was found to be low in group I while values were within normal range in group II and III and high in group IV in pre treatment category. After treatment levels changed significantly towards higher side but within normal range in control and group I and were decreased significantly in group IV while post supplementation levels were found to be towards normal side in group II and III. These findings suggest that chronic fluoride exposure does not have predictable effects on serum hexosamine levels. Studies suggest that breakdown of ground substance by parathyroid hormone (secondary hyperparathyroidism following high fluoride ingestion) is responsible for increased levels of hexosamine in serum (4).

Another ground substance constituent Sialic acid is widely distributed throughout human tissues occurring most abundantly in glycoprotein and glycolipids. It forms important constituent of ground substance. In present study levels of serum sialic acid were low in pre supplementation category in all the studied groups ranging between 4.06-29 mg%. The lowest value was observed in group IV children who were consuming water fluoride greater than 8ppm while highest levels were observed in group I children consuming water having fluoride concentration 1.5 ppm. These findings suggest that levels of serum sialic acid are adversely affected by chronic fluoride ingestion indicating either a decreased synthesis of ground substance of the bones or due to increased renal clearance. Sharma, 1983 (26) also reported a reduction in levels of protein bound sialic acid in rabbits receiving fluoride treatment.

CONCLUSION

From the present study following conclusion can be safely drawn:-

1) Increase in drinking water fluoride concentration results into increased levels of serum fluoride.

2) Increased fluoride intake disturbs normal calcium homeostasis and increases bone resorption evident from increased urinary calcium excretion.

3) Fluorosis disturbs ground substance metabolism as reflected by changes in levels of serum sialic acid, hexosamine and urinary hydroxyproline.

4) Supplementation with ascorbic acid, calcium and vitamin D in appropriate dosage has a definite preventive and a restorative role in children affected with Fluorosis.

BIBLIOGRAPHY

1) WHO. Guidelines for drinking water quality. World health organisation, Geneva 1984, 2:248.

2) PHED Survey report(2001)

3) Rao K, Susheela AK. “Effect of sodium fluoride on adrenal gland of rabbits. Studies on ascorbic acid and delta 5-3 beta hydroxysteroid dehydrogenase activity” Fluoride.1979, 12:67-71

4) Gupta SK. “Environmental health perspective of Fluorosis in children”. Ph.D. Thesis University of Rajasthan, Jaipur, Rajasthan, 1999.

5) Gupta SK, Gupta RC, Seth AK. “Reversal of clinical and dental Fluorosis”. Indian paediatrics. 1994, 31(4): 439

6) Gupta SK, Gupta RC, Seth AK, Gupta A. “Reversal of Fluorosis in children”. Acta Paediatrica Japanica. 1996, 38: 513-519.

7) Fluoride-Ion selective method through Orion model 94-09. Fluoride Alpha, 1989

8) A manual of laboratory techniques by N.Raghuramale, K.Madhawan Nau, K, Kalyansundaram (1983).

9) Varley H, “Determination of Ascorbic acid in blood and plasma”. Practical clinical biochemistry, 4th ed. William Heinemann medical books ltd and Interscience books inc. Newyork. 1975,635-637.

10) OCPC Method (Connerty VH, 1966). Connerty VH & Bruggs RA.

"Determination of serum calcium by means of orthocresopthalein complex". Am. J. Clin Path.1966, 45:290-296.

11) Seibert FB, Plaff ML, Seibert MV. "Serum sialic acid estimation by tryptophane perclonic acid reaction". Arch. Biochem. 1948,18:279.

12) Elson & Morgan. "A colorimetric method for detection of glucosamine and chondrosamine". Biochem.J.1993, 6:1824-1828.

13) Varley H, Gowenloch AH, Belol M. "Determination of Inorganic phosphorus by Gomerri's method. Practical clinical Biochemistry. 5th ed. CBC publishers and distributors. Delhi.1991, 884.

14) Varley H. "Determination of serum alkaline phosphatase" Practical Clinical Biochemistry 4th ed. William Heinmann medical books ltd & Interscience books inc. Newyork.1975, 453-457.

15) Darwin J.Prockop, Karl I. Kivrukko. "Relationship of hydroxyproline excretion in urine to collagen metabolism". Review. Annual of Internal Medicine. 1967, 1243-1266.

16) Juva K, Prockop DJ. "Synthesis of hydroxyproline in vivo by hydroxylation of a polypeptide precursor of collagen". In: Biochemie et physiologie du. Tissu. Conjoctif, edited by Compte P. Societe Ormeco et imprimerie du sud est a lyon, Lyon 1966, pp. 417-432

17) Gottlieb AA, Kaplan A, Unenfriend S. "Further evidence for accumulation of a hydroxyproline deficient, collagenase degradable protein during collagen biosynthesis in vitro". J. Biol. Chem. 1966, 244:1551

18) Barnes MJ, Constable BJ, Morton LF, Kodicek E.: Studies in vivo on biosynthesis of collagen and elastin in ascorbic acid deficient Guinea pigs. Evidence for formation and degradation of a partially hydroxylated collagen: Biochem. J. 1970, 119: 575-85.

19) Barnes MJ, Kodicek E.: Biological hydroxylation and ascorbic acid with special regards to collagen metabolism: Vitam. Harm. 1972, 30: 1-43

20) Schwartz P, Madsen JC, Rasmussen AQ, Transbol IB & Brown EM. "Evidence for a rate of intracellular stored parathyroid hormone in producing hysteresis of PTH calcium relationship in normal humans". Clinical Endocrinology. 1998, 48: 725-732

21) Gupta et al. "Compensatory hyperparathyroidism following high fluoride ingestion". A clinic-biochemical correlation. Indian paediatrics. 2001, 38: 139-146.

22) Grooten HN, Ritskes-Hoitinga J, Mathot JN, Lemmens AG, Beynen AC. "Dietary fluoride prevents phosphorus induced nephrocalcinosis in female rats". Biol. Trace Elem. Res. May 1991, 29(2): 147-55.

23) Bhargavi V, Khandare AL, Venkaiah K, Sarojini G. "Mineral content of water and food in fluorotic villages and prevalence of dental Fluorosis". Biol. Trace Elem. Res. 2004 Sep. 100(3): 195-201

24) Rajyalaxmi K. Technical report, Fluorosis India, International development research centre, Canada.1984.

25) Ming Ho Yu & HUEY Lin Hwang. "Influence of protein and ascorbic acid on fluoride induced changes in blood composition and skeletal fluoride deposition in mice. In: Fluoride Research 1985, studies in environmental sciences". Elsvier Science Publisher BV, Amsterdam. 1988, 27: 203-210.

26) Sharma YD. "Serum sialic acid & ceruloplasmin levels in experimental Fluorosis". Toxicol. Lett. Jan, 1983, 15(1):1-5.