Serum Haptoglobin and C-Reactive Protein in Human Skeletal Fluorosis

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Circulating levels of haptoglobin and C-reactive protein were studied in patients of skeletal fluorosis and compared with two types of controls. The first type of control included normal healthy individuals consuming water containing permissible levels of fluoride (up to 1 mg/L). The second type of control included individuals consuming water contaminated with fluoride (1.2–14.5 mg/L) but not exhibiting clinical manifestations of skeletal fluorosis. A significant increase in the levels of haptoglobin (p < 0.01) and C-reactive protein (p < 0.01) as well as a raised erythrocyte sedimentation rate were seen in patients of skeletal fluorosis as compared to both types of controls. The present study suggests the possibility of a subclinical inflammatory reaction occurring in patients with skeletal fluorosis.

KEY WORDS: haptoglobin; C-reactive protein; inflammation; fluoride.

Introduction

Skeletal fluorosis, a well defined clinical disorder, affects bone (1,2), teeth (3,4), and soft tissues (5–7) adversely. Both cortical and cancellous bones undergo structural and biochemical changes and, in advanced stages of the disease, the changes are irreversible (8,9).

Identifying parameters for early detection of fluoride toxicity/fluorosis is necessary because the disease can be reversed, provided it is detected at an early stage. The sialic acid/glycosaminoglycan ratio (SA/GAG test) in serum thus emerged for early detection of skeletal fluorosis and for differentiating skeletal fluorosis from ankylosing spondylitis (10).

The present report on haptoglobin (Hp) and C-reactive protein (CRP) is yet another effort in identifying other biochemical parameters, if any, closely associated with fluoride toxicity. Hp and CRP are of particular interest because they belong to the group of acute phase proteins. They are synthesized in appreciable amounts following tissue injury, and are used as markers of an inflammatory reaction (11).

Soluble fluorides form hydrofluoric acid in the stomach which is highly corrosive and damages the mucosal lining of the stomach and duodenum (12,13). Fluoride also causes structural damage to muscle fibers (5), and structural and functional derangements in various soft tissues that may be expected to result in an inflammatory reaction.

Fibrinogen, an acute phase protein is reported to be raised in the sera of rabbits treated with sodium fluoride (14).

Mediators of inflammation (IL-1 and IL-6) are known to regulate bone metabolism (15,16) as well as cartilage and chondrocyte metabolism (17). Because fluoride mainly affects bone metabolism (18), it was considered meaningful to investigate the presence of any inflammatory reaction existing in conditions of chronic fluoride toxicity. Hp and CRP have been identified as the parameters for investigation.

Materials and methods

Subjects chosen for study

The study group consisted of 49 patients of which 43 were from the Orthopedic Out-Patient Department and six were inpatients of the All India Institute of Medical Sciences Hospital, New Delhi. All were from areas in which this disorder is endemic and were consuming water containing 1.2–14.5 mg/L fluoride. Two were children: an 8-year-old girl with genu varum who consumed water with 18.0 mg/L fluoride and, a 14-year-old boy with genu valgum who consumed water containing 12.6 mg/L fluoride. Nineteen patients had neurological manifestations such as headache, tingling of the arms, legs, or face, numbness of the fingers/arms, decrease in muscle tone, decrease in anal reflex, and urinary incontinence. One 38-year-old patient with severe sensory deficit also had a complaint of impotence. Eleven patients were totally paralyzed and 13 others could walk only with support. Seven patients who consumed water with 0.4–2.4 mg/L fluoride had

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Manuscript received April 28, 1994; revised July 8, 1994; accepted July 11, 1994.
impaired kidney function; of these, two were 18-
year-old males with genu valgum and two males
were paraplegics. The patients met the following cri-
teria used to establish the clinical diagnosis of flu-
rosis. Each patient had lived in an endemic area for
more than 5 years; had a raised urine and serum
fluoride level; had typical symptoms and findings on
physical examination (Table 1). Gastrointestinal
manifestations are common in fluorosis and help in
confirming the diagnosis of fluorosis (19); in our se-
ries approximately 50% had these manifestations.
Another criteria was that the patient had no evi-
dence of other metabolic bone disorders. All patients
had X-rays of the lumbar spine, forearm, and pelvis.
Radiographs were analyzed by a radiologist of the
hospital. The radiographic features found were an
increased bone density of the spine/pelvic bones
(85%), calcification of the interosseous membranes
or other ligaments (90%). The X-ray of the patient
with genu varum revealed a coarse trabecular pat-
tern and subperiosteal resorption involving the pu-
bic bone, iliac crest, and phalanges.

Definitive confirmation for the diagnosis of flu-
rosis can be made through quantitative measure-
ment of dry ashed bone fluoride. However, this is
invasive and is not required in most cases. Because
the X-ray and physical findings in skeletal fluorosis
are nonspecific, it has been suggested that a com-

bination of raised fluoride levels in urine and serum,
a history of fluoride exposure, X-ray findings, and typ-

ical physical symptoms give conclusive evidence for
the diagnosis of fluorosis (8,20). Two types of con-
trols were investigated. Control 1 were normal
healthy individuals who were living in areas non-
endemic for fluorosis and who consumed water with
fluoride levels less than 1 mg/L. Control 2 were
chosen in such a manner that they were closely related
and living in the same house as the patient, drink-
ing the same water as the patient, but did not ex-
hibit well delineated clinical manifestations of skel-
etal fluorosis. Twenty-three such individuals agreed
to participate in the study.

All individuals who were investigated had no his-
tory of infection, fever, or trauma in the recent past.
None of the patients included had been treated with
drugs affecting acute phase protein levels, namely,
gold, penicillamine, dapsone, corticosteroids, or an-
timalarials. Blood samples from patients and con-
trols were collected between 1100 and 1300 h. Se-

rum was separated and stored at -70 °C. Informed
consent was obtained from all individuals included
in the study.

QUANTITATION OF FLUORIDE

The fluoride content of drinking water collected in
plastic bottles was quantitated on an ION 85 Ion
analyzer (Radiometer, Copenhagen) (21). Fluoride
levels of 24-h urine samples collected in plastic con-
tainers and of sera were also estimated on the Ion
analyzer according to the method of Hall et al. (22).

ESTIMATION OF ERYTHROCYTE SEDIMENTATION RATE (ESR)

The ESR of samples was estimated by the Wes-
tergren method (23), and results reported as the mi-
limeter fall in the first hour.

QUANTITATION OF ALBUMIN IN SERA

Albumin in sera was quantitated on a Genesis au-
toanalyzer and the results reported as g/L of serum.

QUANTITATION OF Hp AND CRP

Reagents

All antibodies used for the Hp and CRP ELISA
were purchased from Dakopatts, Denmark. Bovine
serum albumin (BSA) and Tween-20 were from Sigma
Chemical Company (St. Louis, MO). Sodium
barbitone and o-phenylene diamine were from E.
Merck (Bombay, India) and sodium citrate was pur-
chased from BDH (New Delhi, India). All reagents
used were of analytical grade.

Haptoglobin Elisa

Rabbit anti-human Hp (DAKO A030) diluted 1:
1000 with barbitone buffer (0.05 M, pH 8.8) was
coated in microtiter plates by overnight incubation
at 4 °C. Standard (DAKO X908) and sera from pa-
tients and controls diluted in PBS-T-BSA (phos-
phate buffer, 0.01 M, pH 7.2, containing 0.85% w/v,
NaCl, 0.3%, v/v, Tween 20, and 0.5%, w/v, BSA)
were added in duplicate and the plate incubated at
37 °C for 2 h. Patient and control samples were di-

ulted 10^5-10^6 times in the above buffer. Rabbit anti-
human Hp (DAKO A030) diluted 1:1000 in PBS-T
BSA was then added (2 h at 37 °C). Swine anti-
rabbit IgG (DAKO P217) diluted 1:1000 with PBS-T
(phosphate buffer, 0.01 M, pH 7.2 containing 0.3%,
v/v, Tween 20) was added (2 h at 37 °C) followed by
the substrate o-phenylene diamine in citrate phos-
phate buffer (0.05 M, pH 5.5). The plate was read at
492 nm on a Titertek Elisa reader and the results
expressed as g/L serum.

Table 1

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>No. of Patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low back pain</td>
<td>34 (70)</td>
</tr>
<tr>
<td>Joint pains</td>
<td>35 (71)</td>
</tr>
<tr>
<td>Pain in legs</td>
<td>28 (57)</td>
</tr>
<tr>
<td>Loss of appetite</td>
<td>20 (41)</td>
</tr>
<tr>
<td>Constipation/diarrhea</td>
<td>25 (51)</td>
</tr>
<tr>
<td>Neurological manifestations</td>
<td>19 (39)</td>
</tr>
</tbody>
</table>
**C-reactive protein ELISA**

The method of Highton and Hessian (24) was followed. However, the first antibody (DAKO A 073) and second antibody (DAKO P227) were diluted 1:3000 instead of 1:5000. Patient sera were diluted 5 \times 10^2 times and control sera were diluted 5 \times 10^3 times in PBS (0.01 M, pH 7.2 with 0.85%, w/v, NaCl). The rest of the procedure was the same as described in the original method. The results are expressed as mg/L of serum.

**Statistical calculations**

The data on Hp and CRP was analyzed using a Wilcoxon rank-sum test. Fluoride levels in serum and urine and the ESR data was analyzed using a Kruskal–Wallis test with nonparametric multiple range test. Albumin levels of patients and controls were compared using one-way ANOVA and the multiple-range test.

**Results**

**Fluoride**

Table 2 reveals the data obtained on fluoride content of water, urine, and sera of skeletal fluorosis patients and controls. Serum fluoride levels of patients were raised significantly as compared to control 1 \((p < 0.001)\) and as compared to control 2 \((p < 0.01)\). Serum fluoride levels were also raised significantly in control 2 as compared to control 1 \((p < 0.001)\). Urinary fluoride levels (24 h) of patients were raised significantly as compared to control 1 \((p < 0.001)\) but not as compared to control 2. Urinary fluoride levels of control 2 were however, raised significantly as compared to control 1 \((p < 0.001)\).

**ESR and albumin**

As seen from Table 3 the ESR of male skeletal fluorosis patients was raised significantly as compared to individuals of control 1 \((p < 0.01)\) and control 2 \((p < 0.001)\). The ESR of female fluorosis patients was also raised significantly as compared to individuals of control 1 \((p < 0.01)\) and control 2 \((p < 0.05)\). Circulating albumin levels of fluorosis patients were significantly lower as compared to control 1 and control 2 \((p < 0.05\), in both cases).  

**Hp Assay**

The standard curve for Hp was linear between 1–6 \(\mu g\) Hp/L. The intraassay coefficient of variation (CV) as determined by the estimation of Hp of five samples coated five times each on the same plate was <8%. The interassay CV as estimated by determining Hp concentration of eight samples by coating each sample five times on five different days was found to be <10%.

Hp levels in the sera of patients and controls are shown in Figure 1. The scattergram shows that Hp levels were raised significantly in skeletal fluorosis patients as compared to control 1 \((1.88 \pm 0.64\text{ vs. } 1.39 \pm 0.57\text{ g/L}, p < 0.01)\) and as compared to control 2 \((1.88 \pm 0.64\text{ vs. } 1.18 \pm 0.48\text{ g/L}, p < 0.01)\). Surprisingly, the Hp levels in control 2 subjects were not elevated.

**CRP Assay**

The standard curve for CRP was linear between 2.5–15.0 \(\mu g\) CRP/L. The intraassay CV as determined by the quantitation of Hp of five samples coated five times each on the same plate was <5%.

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**Table 2**  
**Fluoride levels (mg/L)**

<table>
<thead>
<tr>
<th>Skeletal Fluorosis Patients</th>
<th>Control 1</th>
<th>Control 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n) (M, F)</td>
<td>49 (32, 17)</td>
<td>36 (22, 14)</td>
</tr>
<tr>
<td>Water fluoride</td>
<td>4.52 ± 4.03</td>
<td>0.51 ± 0.22</td>
</tr>
<tr>
<td>24-h urine fluoride</td>
<td>3.52 ± 2.76(^a)</td>
<td>0.38 ± 0.21</td>
</tr>
<tr>
<td>Serum fluoride</td>
<td>0.22 ± 0.12(^b)</td>
<td>0.03 ± 0.02</td>
</tr>
</tbody>
</table>

\(^a\) \(p < 0.001\) vs. control 1.  
\(^b\) \(p < 0.01\) vs. control 2.  
\(^c\) \(p < 0.001\) vs. control 1.

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**Table 3**  
**Erythrocyte Sedimentation Rate (ESR, mm Fall in First Hour) and Circulating Albumin Levels (g/L)**

<table>
<thead>
<tr>
<th></th>
<th>Skeletal Fluorosis Patients</th>
<th>Control 1</th>
<th>Control 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ESR in males</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n)</td>
<td>20</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td><strong>ESR in females</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n)</td>
<td>14</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td><strong>Albumin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n)</td>
<td>25</td>
<td>13</td>
<td>10</td>
</tr>
</tbody>
</table>

Values expressed as mean ± standard deviation; \(n\), number of samples.

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controls. The mean Hp and CRP levels were enhanced in skeletal fluorosis patients but not in control 2, that is, individuals from endemic areas not exhibiting clinical manifestations of skeletal fluorosis. The correlation between the levels of Hp and CRP was $r = 0.65$. CRP is an acute-phase reactant (raised up to a 1000-fold), whereas Hp is raised moderately (2-4-fold) during an inflammatory reaction. The normal reference range for these proteins is wide, hence CRP/Hp may be raised several fold over baseline values but still fall in the normal range. The kinetics of the changes in synthesis, secretion, and catabolism of acute phase proteins are complex and depend on factors such as previous injury, sex, hormonal levels (adrenaline, growth hormone, triiodothyronine, and cortisol), and are influenced by such poorly defined phenomena as individual responsiveness (25). This may be the reason for the moderate correlation observed between the two proteins.

In an earlier study on rabbits it was observed that a single high dose of sodium fluoride (20 mg NaF/kg body weight) led to a gradual and significant rise of

The interassay CV as determined by quantitating Hp concentration of five samples by coating each sample three times on five different days was found to be <6%. CRP levels obtained in sera of patients and controls after conversion to the logarithmic form are shown in Figure 2. CRP levels were significantly raised in patients as compared to control 1 ($5.67 \pm 6.95$ vs. $0.66 \pm 0.52$ mg/L, $p < 0.01$) and as compared to control 2 ($5.67 \pm 6.95$ vs. $1.23 \pm 1.15$ mg/L, $p < 0.01$). The correlation coefficient between the Hp and CRP values in patients was $r = 0.65$.

Discussion

The results obtained in the present study reveal that patients with skeletal fluorosis consumed water with high fluoride concentrations as compared to control subjects living in areas nonendemic for fluorosis. The urinary fluoride levels of patients were also raised significantly. Circulating levels of fluoride were also enhanced compared to nonendemic area controls. Individuals living in the same environment and consuming water from the same source as the patients also had raised levels of fluoride in serum and urine as compared to nonendemic area.

Figure 1 — Scatter of Hp levels (g/L) in the sera of skeletal fluorosis patients, control 1, and control 2. Hatched areas represent the normal reference range.

Figure 2 — Scatter of CRP levels (mg/L) in the sera of skeletal fluorosis patients, control 1, and control 2. Hatched areas represent the normal reference range.
Hp levels with a peak at 37–49 h after fluoride administration. Rabbits exposed to 10 mg NaF/kg body weight daily for a period of 18 months, also showed significantly raised Hp levels as compared to rabbits deprived of NaF (unpubl. obsr., Jethanandani). It may be mentioned that fibrinogen, yet another acute phase protein has also been reported to be raised in rabbits treated with NaF (14).

Susheela and Sharma had earlier reported raised levels of the serum mucoid fraction (as hexas and as protein) in the sera of rabbits treated with 50 mg NaF/kg body weight/day for 125 days (26). However, patients of florosis showed a decline of the serum mucoid levels as compared to controls (27).

It is known that during an inflammatory reaction, albumin levels in blood fall and hence albumin is called a negative acute phase protein (28). In the present study it has been found that albumin levels of patients are reduced as compared to those of control 1 and control 2 (p < 0.05 in both cases). This is in agreement with earlier reports on lowered levels of albumin in rabbits administered fluoride (29) and in cattle living in florosis endemic areas (30). Low levels of albumin have also been reported in children suffering from florosis (31) and in florosis patients from the Persian Gulf (32).

Fluoride is a highly reactive ion. In the stomach soluble fluorides give rise to hydrogen fluoride which is highly corrosive and damages the epithelial lining of the gastrointestinal mucosa (6,12,13). Besides, myofibrils and myofilaments of the muscles are also damaged by fluoride (5). Fluoride also damages erythrocytes and induces echinocyte formation (33). These damaged echinocytes are eliminated through the process of phagocytosis. It is possible that tissue damage due to fluoride leads to an inflammatory reaction in order to restore homeostasis. Hp binds to hemoglobin and helps conserve iron and, being a proteinase inhibitor (34) helps prevent tissue damage. Hp is also reported to be an endogenous inhibitor of prostaglandins (35), has immunomodulating properties (36), and plays an important antioxidant role in vivo by preventing the iron-stimulated formation of oxygen radicals (37). CRP in its native cyclic pentameric form is known to bind to damaged tissue, enhances its phagocytosis (38), and thus helps resolve inflammation; in aggregated/proteolyzed forms CRP is proinflammatory (39).

It has recently been reported that Hp enhances bone resorption (40). Raised levels of Hp, CRP, fibrinogen, and other acute phase proteins during an inflammatory reaction are brought about by IL-6, which has been found to be the key mediator of hepatic acute phase protein synthesis (41). IL-1 and IL-6 also have a role in bone metabolism (42–44). Fluoride affects bone metabolism and leads to an uncoupling of bone formation and bone resorption (18). It is possible that fluoride brings about these changes through inflammatory mediators. IL-1 and IL-6 have been implicated in the pathogenesis of bone disorders like rheumatoid arthritis (45) and Paget's disease (46).

Although the exact mechanism involved in bringing about an inflammatory reaction in florosis is yet to be established, the present study on Hp and CRP and the status of albumin, provides convincing evidence to suggest that in skeletal fluorosis, an inflammatory reaction is associated with the disease process. The subjects consuming water with the same amount of fluoride, but not revealing clinical manifestations, need to be explored further in order to understand the biochemical event(s) that precludes these individuals from being afflicted.

References


