Clinical pathological evaluation of leukocytes in patients of fluorosis from Bathinda, Punjab

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INTRODUCTION
Fluoride is naturally present in the earth's crust and can be found in rocks, coal, and clay; thus, it can be found in small quantities in water, air, plants, and animals. Therefore, humans are exposed to fluoride through food, drinking water, and in the air they breathe. Fluorosis is caused by acute or chronic ingestion of fluorides and clinically characterized by dental and musculoskeletal alterations (Foulkes, 2002) as well as changes in hematopoietic organs (Eren et al., 2005).

Since fluoride ion accumulates in bone tissues (Mokezynski and Machoy, 1994), it could conceivably affect the formation of hematopoietic cells in bone marrow cavities and human myeloid and erythroid progenitors (Machalinski et al., 2000). Moreover, fluoride in vitro and in vivo has enhanced generation of superoxide radicals and lipid peroxidation in polymorphonuclear leukocytes and in tissues of fluorosed animals leading to alteration in cell membrane function and structure (Kessabi et al., 1985; Whitford, 1996). Bengtsson et al., (1990) observed aluminium fluoride induction of a pronounced and sustained increase in a filamentous form of actin in intact human neutrophils. Clark (1981) documented that iodination of protein by fluoride stimulated human polymorphonuclear leukocytes and provides evidence that this phenomenon requires both a metabolic burst with the formation of hydrogen peroxide and degranulation. Bober et al., (2000) found that fluoride ion inhibited phagocytosis function of rabbit granulocytes connected with increased degranulation and activated oxygen-dependent bactericidal system in human neutrophils.

Salinas et al., (2010) have demonstrated intake of sodium fluoride as apoptosis inducer in leukocytes of rats treated for eight weeks with 1 or 50 ppm. They suggested that sodium fluoride modifies the expression of p53, bcl-2 and caspase-3 and causes general metabolic changes to leukocytes, which are indicators of changes to normal pattern of apoptosis. The objective of the present work was to describe the effect of fluoride on polymorphonuclear leukocytes in human subjects afflicted with skeletal fluorosis.

MATERIALS AND METHODS
The present study was conducted on 240 patients (118 males, 122 females, and mean age 43.52 ± 14.23 years) affected with clinical dental and skeletal fluorosis living in two highly fluorotic areas viz; Daulatpura and Sivian, district Bathinda, Punjab, India. The levels of fluoride in drinking water varied from 8.05 to 10.25 mg/L (mean

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9.15 ± 1.55 mg/L). The drinking water samples were analysed for fluoride concentration using an electrochemical ion-selective method (Hardwood, 1969).

Blood samples of fluorotic patients were collected into EDTA coated vacutainers. The blood films were stained with Wright and May-Grunwald's Giemsa staining method. Cells were examined under oil immersion by research microscope (Motic). The microphotographs were taken with a digital camera (Motic-480) attached on inverted research binocular microscope. The study was approved by the Institutional ethics committee, Punjabi University, Patiala.

RESULTS AND DISCUSSION
Microscopic images of peripheral blood smear from fluorotic patients revealed many abnormalities in leukocytes. Neutrophils with Pelger-Hüet nucleus were most prominent. A few neutrophils have bilobed peanut shaped nucleus (Figure 1) and dumbbell shaped nucleus (Figure 2). Macrophocytes showed increased nuclear segmentation (Figure 3) due to defect in the production of DNA which causes the maturation process to be slower than normal that in turn causes the nucleus to hypersegment. In some neutrophils, the nucleus has 7 lobes, separated into fragments and not connected by thin filaments of chromatin. Another rounded nuclear fragment was located in cytoplasm of neutrophil (Figure 4). Abnormal neutrophils with ring or doughnut nuclei were visible (Figure 5).

Some neutrophils have nonsegmented rounded nucleus with very coarse chromatin clumping (Figure 6). Necrobiotic neutrophils have dense homogenous three pyknotic nuclei (Figure 7) or four pyknotic nuclei (Figure 8). The nuclear sticks were increased in size in granulocytes (Figure 9). Macro-neutrophils have abnormal hook like nuclear projections (Figure 10) and showed extensive vacuolization (Figure 11). Small dark blue to purple granules resembling primary granules appeared in the cytoplasm of neutrophils accompanied by the presence of vacuoles in the cytoplasm which represents a transient change in the cytoplasmic morphology of neutrophils due to toxic effect of fluoride (Figure 12).

Reduced granulation in neutrophil cytoplasm alongwith pyknotic nucleus was present. Dark blue deeply stained clumped nuclear chromatin was observed (Figure 13). A few cells exhibited neutrophil aggregation and clumps (Figure 14). The eosinophils revealed nuclear hypersegmentation and abnormal granulation with irregularly shaped outer cytoplasmic membrane. The nuclei were trilobed (Figure 15). Hypergranulation (Figure 16), finger like cytoplasmic projections (Figure 17), distorted and accentric nuclei (Figure 18) were prominent alterations seen in eosinophils.

The granulated lymphocytes have irregularly shaped condensed nuclear chromatin material. The cytoplasm was abundant and contained coarse azurophilic granules visible in Wright-Giemsa preparations (Figure 19). Instead of round or oval shaped lymphocytes, some lymphocytes became spindle shaped. These cells have oval basal nucleus eccentrically located in light-blush cytoplasm.

The characteristic feature of cytoplasm was its long tapered tail. A deeply stained dark blue nucleus having a smooth surface with dense nuclear chromatin was present (Figure 20). Abnormal lymphocytes have villous cytoplasmic projections (Figure 21). A few lymphocytes with a cleavage in the center of nucleus appeared (Figure 22). Vacuolated lymphocytes contained nuclear vacuoles (Figure 23) and cytoplasmic vacuoles with moderate amount of intensely basophilic cytoplasm (Figure 24). An enlarged dysmorphic downey cell lymphocyte was characterized by having monocyte-like nucleus.

The cytoplasm was found to have an irregular border giving scalloped appearance and was increased in quantity (Figure 25) which stretched the length of the cell, with chromatin strands that parallel the length of the nucleus and appeared almost blast-like. The cytoplasm showed light staining around the nucleus but more intense blue color at the periphery (Figure 26). In some lymphocytes the nucleus was "shifted to the right side" with clear nuclear outline (Figure 27).

However, the nucleus in other lymphocytes, was "shifted to the left side" which indicates the presence of precursor of granulocytes (Figure 28). The apoptotic lymphocytes were abundant. These cells were recognized by peripheral condensation of the nucleus and a glary appearance of the cytoplasm (Figure 29).

The present study demonstrates disturbances in granulocytes and agranulocytes morphology in patients exposed to 8.05 to 10.25 mg fluoride per liter in drinking water. The study presented morphological evidence for degranulation in the neutrophil cytoplasm alongwith pyknotic nuclei. The cells exhibited less cytoplasmic granularity than normal cells. Degranulation comprises the emptying of the contents of the cytoplasmic granules into the vacuoles and to the outside of the cell. The findings are in consonance with the study of Gabler et al., (1989) which reported fluoride induced degranulation of both primary and specific granules in humans.

Niessen et al., (1994) had demonstrated that aluminium fluoride induce several biochemical and functional
responses including activation of degranulation and exocytosis in intact human neutrophils. In experimental animals, the degranulation in polymorphonuclear leukocytes has been attributed to release of cytoplasmic enzymes like lactate dehydrogenase, alkaline phosphatase, acid phosphatase (Elferink et al., 1980).

Bober et al., (2000) have explained that fluoride ion inhibited phagocyte functions of rabbit granulocytes connected with increased degranulation. In the present study, destruction of membrane induced by fluoride toxicity in polymorphonuclear leukocytes results in strong exocytosis and cytolysis with formation of apoptotic neutrophils, neutrophil with nuclear vacuolization, hypergranulation, cleaved lymphocytes and downey cell lymphocytes. Exocytosis may be due to either to an interaction of fluoride with inner side of the membrane or to a reaction with a specific membrane component. In some cases of fluorosis, hypersegmented neutrophils with six nuclear lobes were seen. The extra lobes are due to the defective DNA production. The defect shows maturation thus producing many segments.

In our study, we have found apoptotic leukocytes. It is known that leukocytes in peripheral blood circulation that have completed their life present a natural process known that leukocytes in peripheral blood circulation afflicted with fluorosis. In our study, we have found apoptotic leukocytes. It is morphological alterations in leukocytes in patients producing many segments. The extra lobes are due to the defective DNA production. The defect shows maturation thus producing many segments.

In our study, we have found apoptotic leukocytes. It is known that leukocytes in peripheral blood circulation that have completed their life present a natural process of apoptosis and are eliminated from the organism (Machalinska et al., 2001). Determination of the general metabolic activity of leukocytes has been considered an indicator of the normal functioning state of this type of cell. The patients of fluorosis are more susceptible to presenting bacterial, viral, and parasitic infections, which indicates that there can be a change in their immune system, in which leukocytes play a very important role as defense systems against these infections (Foulkes, 2002). Moreover, decrease in the metabolic activity of leukocytes treated with 300 ppm sodium fluoride for 10 weeks has been reported (Otsuki et al., 1991).

Experiments in vitro have demonstrated that leukocytes incubated in the presence of sodium fluoride present an increase in the expression of proteins p53 and bcl-2, which are considered markers of the presence of apoptosis due to cellular damage. Protein p53 has been described as an indicator of DNA damage which is expressed after exposure to a toxin (Morales-Gonzalez et al., 2004; Bogatcheva et al., 2006). Salinas et al., (2010) reported an increase in the expression of proteins p53 and caspase-3 in the group of rats that received a 50-ppm dose of sodium fluoride indicates an increase of apoptosis in leukocytes. Our study has confirmed that fluoride is one of the chemical substances responsible for producing different types of morphological alterations in leukocytes in patients afflicted with fluorosis.

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REFERENCES


Microphotographs of peripheral blood smear of fluorotic patients showing abnormalities in neutrophils:

**Figure 1:** Neutrophil with Pelger-Hüt round nucleus. Wright-Giemsa stain, (X1000).

**Figure 2:** Neutrophil with Pelger-Hüt dumbbell nucleus. May Grunwald Giemsa stain, (X1000).

**Figure 3:** Macropolyocyte. Wright-Giemsa stain, (X1000).

**Figure 4:** Neutrophil showing Hypersegmentation. May Grunwald Giemsa stain, (X1000).

**Figure 5:** Neutrophil with ring shaped nucleus. Wright-Giemsa stain, (X1000).

**Figure 6:** Neutrophil with non segmented rounded nucleus. Wright-Giemsa stain, (X1000).

**Figure 7:** Necrobiotic neutrophil. Wright-Giemsa stain, (X1000).

**Figure 8:** Necrobiotic neutrophil. May Grunwald Giemsa stain, (X1000).

**Figure 9:** Neutrophil with increased nuclear sticks. May Grunwald Giemsa stain, (X1000).

**Figure 10:** Macro-neutrophil with abnormal hook like nuclear projections. Wright-Giemsa stain, (X1000).

**Figure 11:** Neutrophil with nuclear vacuolization. Wright-Giemsa stain, (X1000).

**Figure 12:** Toxic granulation. May Grunwald Giemsa stain, (X1000).

**Figure 13:** Neutrophil Hypogranulation. May Grunwald Giemsa stain, (X1000).

**Figure 14:** Clumps of neutrophils. Wright-Giemsa stain, (X1000).

Microphotographs of peripheral blood smear of fluorotic patients showing abnormalities in eosinophils:

**Figure 15:** Hypersegmented eosinophil with trilobed nuclei. Wright-Giemsa stain, (X1000).

**Figure 16:** Hypergranulated eosinophil. Wright-Giemsa stain, (X1000).

**Figure 17:** Eosinophil with cytoplasmic finger like projection. Wright-Giemsa stain, (X1000).

**Figure 18:** Eosinophil with eccentric nuclei. Wright-Giemsa stain, (X1000).

Microphotographs of peripheral blood smear of fluorotic patients showing abnormalities in lymphocytes:

**Figure 19:** Granulated lymphocyte. Wright-Giemsa stain, (X1000).

**Figure 20:** Spindle shaped lymphocyte. May Grunwald Giemsa stain, (X1000).

**Figure 21:** Villous lymphocyte. Wright-Giemsa stain, (X1000).

**Figure 22:** Cleaved lymphocyte. May Grunwald Giemsa stain, (X1000).

**Figure 23:** Lymphocyte with round vacuole in nucleus. Wright-Giemsa stain, (X1000).

**Figure 24:** Lymphocyte with cytoplasmic vacuoles. Wright-Giemsa stain, (X1000).

**Figure 25:** Downey cell showing Scalloped appearance. May Grunwald Giemsa stain, (X1000).

**Figure 26:** Downey cell showing blast like appearance. Wright-Giemsa stain, (X1000).

**Figure 27:** Neutrophil nucleus shift to right. May Grunwald stain, (X1000).

**Figure 28:** Neutrophil nucleus shift to left. May Grunwald Giemsa stain, (X1000).

**Figure 29:** Apoptotic lymphocyte. Wright-Giemsa stain, (X1000).