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Research Article

FLUORIDE INDUCED TOXIC EFFECT ON CASPASE3 PROTEIN FORMING GENE IN SWISS ALBINO MICE

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ABSTRACT
It has been suggested that oxidative stress plays a major role in various forms of cell death, including necrosis and apoptosis. We have previously reported that fluoride (NaF) induces apoptosis in HL-60 cells by caspase-3 activation. The main focus of this investigation was to arrive at a possible pathway of the apoptosis induced by NaF upstream of caspase-3, because the mechanism is still unknown. The present study showed that after exposure to NaF, there was an increase in MDA and 4-HNE and a loss of mitochondrial membrane potential (deltaPsi (m)) was also observed in NaF-treated cells. Fluoride (NaF) induces apoptosis in HL-60 cells by caspase-3 activation. There was a significant increase in cytosolic cytochrome c, which is released from the mitochondria. We have reported a downregulation of Bcl-2 protein in NaF-treated cells. The antioxidants N-acetyl cysteine (NAC), glutathione (GSH) protected the cells from loss of deltaPsi(m), and there was no cytochrome c exit or Bcl-2 downregulation, and we suggest that these antioxidants prevent apoptosis induced by NaF. These results suggested that perhaps NaF induced apoptosis by oxidative stress-induced lipid peroxidation, causing loss of deltaPsi(m), and thereby releasing cytochrome c into the cytosol and further triggering the caspase cascade leading to apoptotic cell death in HL-60 cells.

***Key Words**: Fluoride, Toxicity, Gene, Caspase3, Albino Mice*

INTRODUCTION
Fluorosis caused by excess intake of fluoride is a slow, progressive degenerative disorder, known to affect predominantly the skeletal systems, teeth and also the structure and function of skeletal muscle, brain and spinal cord. Recent studies have shown accumulation of fluoride in the hippocampus of the brain causing degeneration of neurons and decreased aerobic metabolism and altered free-radical metabolism in the liver, kidney, and heart. However, the effect of fluoride on neuromuscular tissue is far from clear. Hydropic degeneration in lamina propria of intestine and muscular tissue, increase in the number of goblet cells, broken tips of villi, nuclear pyknosis, and abnormal mitosis were observed (Sondhi *et al.,* 1995). In high concentrations, soluble fluoride salts are toxic and skin or eye contact with high concentrations of many fluoride salts is dangerous. Referring to a common salt of fluoride, NaF, the lethal dose for most adult humans is estimated at 1–10 grams A lethal dose is approximately 28 mg per kilogram of body mass. hosphatidyicholine and sphingomyelin remained unaltered during NaF ingestion, but a significant decrease occurred in phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. Significantly low levels of glutathione after 30 days of treatment were also obtained. On comparing the alterations in protein profile, phospholipids and glutathione in both tissues, it was evident that the protein profile was disturbed more in testis than in cauda epididymis, whereas phospholipids and glutathione levels were affected more in cauda than in testis (Chinoy *et al.,* 1997). Epididymal sperm count was decreased significantly in the fluoride-treated group and qualitative examination of testicular sections revealed fewer mature luminal spermatozoa in comparison to the control. The seminiferous tubules were dilated in treated animals. Fluoride treatment was associated with oxidative stress as indicated by an increased level of conjugated dienes in the testis, epididymis, and epididymal sperm pellet with respect to control. Peroxidase and catalase activities in the sperm pellet were decreased significantly in comparison to the control (Ghosh *et al.,* 2002).

Caspase 3 is a caspase protein which interacts with caspase 8 and caspase 9.This gene encodes a protein that is a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspases exist as inactive proenzymes that undergo proteolytic processing at conserved aspartic residues to produce two subunits, large and small, that dimerize to form the active enzyme. This protein cleaves and activates caspases 6, 7, and 9; and the protein itself is processed by caspases 8, 9, and 10. It is the predominant caspase involved in the cleavage of amyloid-beta 4A precursor protein, which is associated with neuronal death in Alzheimer's disease. Alternative splicing of this gene results in two transcript variants that encode the same protein. The ladder formation was observed when DNA isolated from fluoride-exposed alveolar macrophages was electrophoresed in agarose gel. These results suggest that cytotoxicity of fluoride is associated with apoptosis in rat alveolar macrophages (Seishiro *et al.,* 1996). A significant reduction in electrolyte levels of sperm also occurred which would also affect their viability. The protein levels in cauda epididymal sperm suspension, vas deferens, seminal vesicle and prostate were significantly decreased after NaF administration, which may be due to altered protein metabolism by interference of fluoride ions. The changes in epididymal protein profile, with absence of some proteins and induction of some new ones, were probably a result of the "stress proteins" in NaF-treated rats affecting the structural and functional integrity of sperm (Chinoy *et al.*, 1995).

Most of the following reversible ill effects caused by fluoride were first recognized among aluminum workers in the 1930s by the Danish health officer Dr. Kaj Roholm. Not all the Symptoms are necessarily present at the same time. Their severity and duration(often episodic) depend on a person's age, nutritional status, environment kidney function amount of fluoride ingested' genetic background tendency to allergies and other factors.To test for fluoride intoxication, the following procedure must be rigorously followed Avoid all fluoridated water (substitute distilled or other nonfluoridated low-fluoride water), fluoridated Beverages, fluoride-rich foods (tea, ocean fish, gelatin, skin of chicken,etc.), fluoridated toothpastes and any other source of environmental fluoride,including cigarette smoke and industrial pollution.

Fluoride is an essential trace element in humanbodies and is highly correlated with the metabolism of bone and tooth. But excessive exposure to fluoride for a long term leads to bone damage with complicated pathological changes such as osteoporosis and osteopetrosis (FACCINI, 1969). Arsenide is an environmental toxicant and a known carcinogen Inorganic arsenide is highly accumulated *in vivo*. Arsenate deposits in skeleton because it takes the place of phosphate in the apatite crystal of bone. Fluoride and arsenide in groundwater, air, and food in some areas of China as a result of specific geographical and geological environment and living habits of local people have tremendous impact on health of local residents. Great attention has been paid to the possible combined effect of fluoride and arsenide.There are different reports about the role of arsenide in the bone damage caused by fluoride. Both osteoblasts and osteoclasts are involved in bone damage. Studies indicate that the proliferation, differentiation, and maturity of osteoclasts are dependent on the existence of osteoblasts. Osteoprotegerin (OPG) and osteoclast differentiation factor (ODF) are secreted by osteoblasts. The former is responsible for inhibiting osteoclastogenesis, while the latter plays an important role in stimulating the differentiation and maturity of osteoclasts, activating mature osteoclasts and inhibiting their apoptosis. So OPG and ODF may combine the functions of osteoblasts and osteoclasts, which is of great help to the study of the influence of toxicants on bone remodeling. A study showed that after exposure to NaF, there was an increase in MDA and 4-HNE and a loss of mitochondrial membrane potential (deltaPsi(m)) was also observed in NaF-treated cells. Fluoride (NaF) induces apoptosis in HL-60 cells by caspase-3 activation (Anuradha *et al.,* 2001). There was a significant increase in cytosolic cytochrome c, which is released from the mitochondria.

Poisoning comes from ingesting a large amount of fluoride in a short period of time. Ingesting 3–5 mg/kg may cause symptoms to appear, while the estimated lethal dose is 5–10 g (32–64 mg/kg) in adults and 16 mg/kg in children. Severity of symptoms depends upon the amount of fluoride ingested. These include

abdominal pain, diarrhea, dysphagia, hypersalivation, mucosal injury, nausea, vomiting. Electrolyte abnormalities including hyperkalemia, hypocalcemia, hypoglycemia, and hypomagnesemia may occur. Neurological symptoms include headache, muscle weakness, hyperactive reflexes, muscular spasms, paresthesia seizures, tetanic contractions, and tremors. In severe cases, multi organ failure will occur. Death typically results from cardiac arrest, shock, widening of QRS, and various arrhythmias occur The only generally accepted adverse effect of fluoride at levels used for water fluoridation is dental and skeletal fluorosis. Which can alter the appearance of children's teeth during tooth development; this is mostly mild and not usually of aesthetic concern. Compared to unfluoridated water, fluoridation to 1 mg/L is estimated to cause fluorosis in one of every 6 people (range 4–21), and to cause fluorosis of aesthetic concern in one of every 22 people (range 13.6–∞). Here, "aesthetic concern" is a term used in a standardized scale based on what adolescents would find unacceptable, as measured by a 1996 study of British 14-year-olds.

Caspases, or *c*ysteine-*asp*artic prote*ases*, are a family of cysteine proteases, which play essential roles in apoptosis (programmed cell death), necrosis and inflammation. Caspases are essential in cells for apoptosis, or programmed cell death, in development and most other stages of adult life, and have been termed "executioner" proteins for their roles in the cell. Some caspases are also required in the immune system for the maturation of cytokines. Failure of apoptosis is one of the main contributions to tumour development (Dias *et al.*, 2000) and autoimmune diseases; this coupled with the unwanted apoptosis that occurs with ischemia or Alzheimer's disease, has stimulated interest in caspases as potential therapeutic targets since they were discovered in the mid 1990s. Recent indications suggest the involvement of homeobox genes in (i) crucial adult eukariotic cell functions and (ii) human diseases, spanning from diabetes to cancer (Cillo *et al.*, 2001).

As of November 2009, twelve caspases have been identified in humans. There are two types of apoptotic caspases: initiator (apical) caspases and effector (executioner) caspases. Initiator caspases (e.g. CASP2, CASP8, CASP9 and CASP10) cleave inactive pro-forms of effector caspases, thereby activating them. Effector caspases (e.g. CASP3, CASP6 and CASP7) in turn cleave other protein substrates within the cell, to trigger the apoptotic process. The initiation of this cascade reaction is regulated by caspase inhibitors.CASP4 and CASP5, which are overexpressed in some cases of vitiligo and associated autoimmune diseases caused by NALP1 variants(Anuradha et.al.,2001) are not currently classified as initiator or effector in MeSH(Carlson et.al.,1960), because they are *inflammatory* enzymes which, in concert with CASP1, are involved in cytokine maturation. CASP14 is not involved in apoptosis or inflammation, but instead is involved in skin cell development.Caspase 3 is a caspase protein which interacts with caspase 8 and caspase 9.This gene encodes a protein that is a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspases exist as inactive proenzymes that undergo proteolytic processing at conserved aspartic residues to produce two subunits, large and small, that dimerize to form the active enzyme. This protein cleaves and activates caspases 6, 7, and 9; and the protein itself is processed by caspases 8, 9, and 10. It is the predominant caspase involved in the cleavage of amyloid-beta 4A precursor protein, which is associated with neuronal death in Alzheimer's disease. Alternative splicing of this gene results in two transcript variants that encode the same protein.

MATERIAL AND METHODS

Twelve healthy, adult female albino mice, *Mus musculus* of Swiss strain, each weighing about 30 ± 2 g, were obtained from the Animal house. *Body weight and organo-somatic index:* The body weight of each animal was noted before treatment and also on day 15. The weight of liver of respective groups of animals was recorded after dissection tissue samples were stored at -20c freezer and before DNA extraction samples from freezer were kept on ice.

Procedure

Chopped small amount of tissue (as finely as you can) with a sterile scalpel blade.
Taken Approximately 200 µl chopped sample tissue in a 2 ml eppendorf tube.
Added 600 µl of TNES buffer and 35 µl of Proteinase-K (20 mg/ml) and Mixed the sample by inverting the tubes several times.
Tubes were incubated at 55°c overnight.
Remove tubes from oven and carefully add 800 µl PCI (phenol: chloroform: IAA).
Carefully removed aqueous layer for each sample into new, clean microfuge tube.
Add 45ul of 3M NaAC pH 5.3 to each tube. Closed tubes and Mixed properly, then added approx. 100 µl Ethanol (100%).
Invert to mix and precipitate DNA and kept in -20c freezer for 30 minutes.
Spin at 12,000rpm for 10 min at 4ºC.
Removed supernatent to a new, labeled 2 ml microcentrifuge tube.
Added an equal volume of cold 100 % ethanol and gently mix by inverting the tubes.
Centrifuged the samples at 12,000 rpm for 15 minutes at 4ºC.
Remove the supernatent and wash the DNA pellet in 500 µl of 100 % ethanol (add ethanol, close cap of tube and invert gently).
Pour (or pipette) off the ethanol and briefly spin the samples to keep the pellet at the bottom of the tube.
Washed DNA pellet with 70 % ethanol as above. After removing the 70 % ethanol, briefly
Centrifuge the samples to get the last of the ethanol to the bottom of the tube; pipette off the remaining ethanol.
The sample was to air dried for 1-3 hr depending upon the temperature.
Re-suspended the dried DNA sample into 100-200 µl of sterile distilled water or Tris-EDTA Buffer.
6 M NaCl is saturated salt solution stored at 37ºC: weigh out 6 M NaCl and heat the solution until it dissolves. Leave to cool at room temperature. Some crystals will form, this is normal!
After isolation of DNA, its quantification and analysis of quality are necessary to ascertain the approximate quantity of DNA obtained and the suitability of DNA sample for further analysis. This is important for many applications including digestion of DNA by restriction enzymes or PCR amplification of target DNA.
The 260/280 ratio should range from 1.6-1.9 for preparations of DNA that are to be used for PCR amplification. If there is contamination with protein or phenol, the ratio will be significantly less than 1.8 and if ratio is greater than 2.0, there can be possible contamination of RNA. Accurate quantitation of amount of nucleic acid will not be possible in such a sample and it cannot be used for further processes.
Agarose gel electrophoresis was used twice; once after PCR reaction to confirm the amplicon size.

Caspase 3, apoptosis-related cysteine peptidase (CASP3)

Table 6: PCR cycling conditions for CAS

Steps		Temperature	Time	No of Cycles
Step I	Initial Denaturation	96°C	1 min	1 cycle
	Denaturation	96°C	60 Sec	38 cycles
	Annealing			
Step II		54°C	60 Sec	
	Extension	72°C	120 Sec	
	Final Extension			1 cycle
Step III		72°C	5 min	
Hold at 4°C				

RESULTS AND DISCUSSION

The cycle conditions were standardized before starting with amplification of test samples. The amplification was studied at different annealing temperatures and suitable annealing temperature was selected for final reaction setup. The amplification was observed for the entire test DNA samples.

Caspases, or cysteine-aspartic proteases (CASP3).

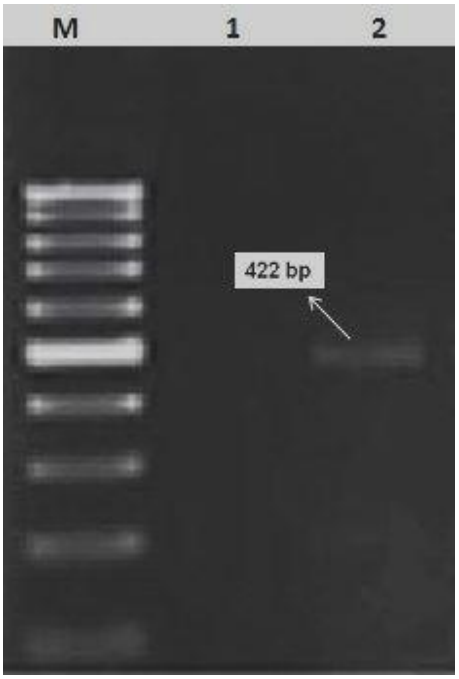

Figure 1: **PCR product run on 2.0 % agarose gel and was stained with EtBr (10 mg/ml). Lane M: 100 bp DNA Ladder; Lane 2: PCR product.**

The Albino mice have been subjected to fluoride intoxication for 14 days by administering the dose of aqueous NaF (20 mg/kg/body weight/day). At the end of the 14 day treatment, the animals were sacrificed by cervical dislocation, and the liver is dissected out, blotted free of blood, transferred to trays maintained at ice-cold conditions and used for isolation of DNA. Genomic DNA was isolated using standardized protocol and quantified on spectrophotometer to check its quality and then run on 8 % gel.

Methods validated for the PCR Amplification and run for the four genes under study. The amplified PCR products were run on 2% Agarose gel with the 100 bp DNA ladder the check the amplified product size. The results obtained for the Caspase gene under study did not vary for the normal and test mice; we get the same size of amplified products for both the cases. The PCR products obtained were further sequenced and matched but there was not any difference in their sequences.

In conclusion, our findings revealed that we need to elaborate our study with more genes thats can be influenced and damaged by different forms of fluorine compounds. With our results and studies we assume may be substantially more evident effect was caused by other fluoride compounds compared to simple fluoride ion released by sodium fluoride.

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