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Research Article

EFFECT OF FLUOROSIS ON BCL-2 PROTEIN FORMING GENE IN SWISS ALBINO MICE

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ABSTRACT
India is one of the worst fluorosis affected countries, with large number of people suffering. This is because a large number of Indians rely on ground water for drinking purposes and water at many places is rich in fluoride. Pathological effects of fluoride intake have been reported for various organs including the brain, kidney, liver and thyroid of humans living in areas with areas with endemic fluorosis in several developing countries, including China and India. In experimental animals subjected to chronic fluorosis, including swelling of mitochondria, dilation of the endoplasmic reticulum and frequent apoptosis. Interestingly, the apoptosis induced by fluorosis has been recently focused on as a possible toxicological mechanism for the disease. Integrating mitochondrial fragmentation, the procedure of apoptosis associated with chronic fluorosis might be involved in the mitochondria-mediated pathway regulated by anti-apoptotic Bcl-2 families. The mitochondrial pathway is initiated y the release of cytochrome c from the mitochondria into the cytosol, which is controlled by Bcl-2 family. The study was conducted to find the effect of fluorosis on apoptosis and the expression of Bcl-2 in liver of Swiss Albino mice in an attempt to elucidate molecular mechanisms.

Keywords: *Fluorosis, Apoptosis, Anti-apoptotic Bcl-2 Families*

INTRODUCTION
Fluorosis is an important public health problem in 24 countries, including India, which lies in the geographical fluoride belt that extends from Turkey to China and Japan through Iraq, Iran and Afghanistan (Saravanan *et al.*, 2008). Of the 85 million tons of fluoride deposits on the earth's crust, 12 million are found in India (Teotia *et al.*, 1984) Hence it is natural that fluoride contamination is widespread, intensive and alarming in India. 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). Endemic fluorosis resulting from high fluoride concentration in groundwater is a public health problem in India (Kotecha *et al.*, 2012).
The available data suggest that 15 States in India are endemic for fluorosis (fluoride level in drinking water >1.5 mg/l), and about bout 62 million people in India suffer from dental, skeletal and non-skeletal fluorosis. Out of these; 6 million are children below the age of 14 years (Susheela, 2001). Groundwater is considered as the major source of drinking water in most places on earth (Brindha *et al.*, 2011).
It has long been known that excessive fluoride intake carries serious toxic effects on skeletal muscle, brain and spinal cord. Recent studies have shown accumulation of fluoride decreases aerobic metabolism and altered free-radical metabolism in the liver, kidney and heart (Sondhi *et al.*, 1995). It has been accepted that fluorides chromosomal aberrations and gene mutations in cultured mammalian cells (Charles *et al.*, 1998). Sodium fluoride, the first and still-recommended fluoride compound used for fluoridation of drinking water, induces morphological and neoplastic transformation of Syrian hamster embryo cells (Jones *et al.*, 1988a; Lasne *et al.*, 1988). Sodium fluoride has also been observed to have tumour-promoting activity in these cells (Jones *et al.*, 1988b). Cells undergoing apoptosis shows distinct morphological and biochemical changes such as cell shrinkage, membrane blabbing, chromatin condensation and DNA fragmentation (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). The apoptotic events are thought to be mediated by the caspase family of proteases which cleave death substrates (Enari *et al.*, 1996; Liu *et al.*, 1997; Rudel and Bokoch, 1997). Interestingly, the apoptosis induced by fluorosis has been recently focused on as a possible toxicological mechanism for the disease. Integrating mitochondrial

fragmentation, the procedure of apoptosis associated with chronic fluorosis might be involved in the mitochondria-mediated pathway regulated by anti-apoptotic Bcl-2 families. The mitochondrial pathway is initiated y the release of cytochrome c from the mitochondria into the cytosol, which is controlled by Bcl-2 family (Ankarcrona *et al.*, 1995).

Consistently, researchers from numerous different disciplines (physiology, toxicology, medicine, dentistry, public health, nutrition) have lines of evidence and analysis. Scientists are now debating whether fluoride confers any benefit at all. The study was conducted to find the effect of fluorosis on apoptosis and the expression of Bcl-2 in liver of Swiss Albino mice in an attempt to elucidate molecular mechanisms. The sequencing of PCR products of the gene Bcl-2 of the normal and treated mice would contribute to a better understanding of the mechanism of fluoride effect in the cellular system.

MATERIAL AND METHODS

Animal source: Adult female Swiss albino mice, *Mus musculus*.

Reagents: 0.9% saline solution, Aqueous NaF Solution.

Six healthy, adult female albino mice, *Mus musculus* of Swiss strain, each weighing about 30 ± 2 g, were obtained from the Animal house. They were caged with allowed to acclimatize to the laboratory conditions for four days before experiments began. Meanwhile animals were fed a standard pellet diet and given distilled water. The body weight of each animal was noted before treatment and also on day 15. They will be offered fluoride diet for 14 days by administering the dose of aqueous NaF (20 mg/kg/body weight/day) to monitor their genetic effects. At the end of the 14 day treatment, the animals were sacrificed by cervical dislocation, and the liver is dissected out, blotted weighed and transferred to 0.9% saline solution. Samples were maintained at ice-cold conditions and used for isolation of genomic DNA.

Material for DNA Extraction

Source: Liver tissue.

Reagents: 0.9% saline solution, TNES buffer, 35 µl of Proteinase-K (20 mg/ml), PCI (phenol: chloroform: IAA), 3M NaCl, 70 % and 100% Ethanol, Tris-EDTA Buffer.

Instruments & Equipments: Micro-Centrifuge.

Other requirement: Sterile Scalpel, Petri dishes, Eppendorff, Micropipettes, Sterile Micro-tips, Freezer, Cello tape, Gloves, Apron, Filter paper, Cotton, etc.

Material for PCR

Source: Extracted DNA.

Reagents

Taq DNA polymerase Buffer, dNTP's (dATP, dGTP, dCTP, dTTP)**,** Forward primer, Reverse primer, Taq DNA polymerase, Ethidium bromide. Agarose, Gel loading Buffer, DNA ladder, Autoclaved distilled water, Alcohol, etc.

Instruments & Equipments

Laminar flow, Thermo cycler (Make-Applied Biosystems), Electrophoresis unit, UV-trans-illuminator, Microwave, Weighing balance, power pack etc.

Other Requirement

PCR tubes, Micropipettes, Sterile Micro-tips, Cello tape, Gloves, Apron, Filter paper, Cotton, etc.

Material for DNA Quantification

Source: Extracted genomic DNA sample.

Reagents

TE buffer, Autoclaved distilled water

Instruments & Equipments

Laminar flow, UV spectrophotometer Microwave, Weighing balance, power pack etc.

Other Requirement

Quartz cuvettes, Micropipettes, Sterile Micro-tips, Cello tape, Gloves, Apron, Filter paper, Cotton, etc.

Procedure

I. DNA Extraction

Procedure

1. Finally chopped liver tissue with a sterile scalpel blade.
2. Homogenized in 0.9% saline solution.
3. Taken Approximately 200 µl chopped sample tissue in a 2 ml eppendorf tube.
4. 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.
5. Tubes were incubated at 55°C overnight.
6. Remove tubes from oven and carefully add 800 µl PCI (phenol: chloroform: IAA).
7. Carefully removed aqueous layer for each sample into new, clean microfuge tube.
8. Add 45 µl of 3M NaCl pH 5.3 to each tube. Closed tubes and Mixed properly and then added approx. 100 µl Ethanol (100%).
9. Invert to mix and precipitate DNA and kept in -20c freezer for 30 minutes.
10. Spin at 12,000rpm for 10 min at 4ºC.
11. Removed supernatant to a new, labeled 2 ml micro centrifuge tube.
12. Added an equal volume of cold 100 % ethanol and gently mix by inverting the tubes.
13. Centrifuged the samples at 12,000 rpm for 15 minutes at 4ºC.
14. Remove the supernatant and wash the DNA pellet in 500 µl of 100 % ethanol (add ethanol, close cap of tube and invert gently).
15. Pour (or pipette) off the ethanol and briefly spin the samples to keep the pellet at the bottom of the tube.
16. Washed DNA pellet with 70 % ethanol as above. After removing the 70 % ethanol, briefly.
17. Centrifuge the samples to get the last of the ethanol to the bottom of the tube; pipette off the remaining ethanol.
18. The sample was to air dried for 1-3 hr depending upon the temperature.
19. Re-suspended the dried DNA sample into 100-200 µl of sterile distilled water or Tris-EDTA Buffer.

II. PCR Setup

DNA samples: DNA extracted from test samples using standard protocol.

Primers: The details of primers obtained from axygen:

Table 1: Primer sequences with product sizes

Sr. No.	Genes	Primers sequence	Amplicon size
1.	BCL-2	Forward primer: 5′-TAC CGT CGT GAC TTC GCA GAG -3′ Reverse primer: 5′-GGC AGG CTG AGC AGG GTC TT -3′	350 base pair (bp)

1. Before starting with the reaction set-up, the sample DNA was diluted with HPLC water to make it 10µg/µl. Primers, both forward and reverse were diluted in the ratio 1:10. Prepared the reaction mixture for PCR by adding the reagents given in the kit as given in table:

Table 2: ?????

Sr. No.	Materials	Amount (µl)	Dilution
1.	AampliTaq Gold PCR Master Mix	10	-
2.	HPLC water	3.0	-
3.	DNA (10 ng / µl	6.0	-
4.	Forward primers	0.5	1:10
5.	Reverse primers	0.5	1:10

2. Carried out the amplification using the above mentioned conditions and performed at least 302cycles for amplification of the DNA fragment.

PCR Cycling Conditions for BCL-2 Gene

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.

Steps		**Temperature**	**Time**	**No of Cycles**
Step I	Initial Denaturation	94°C	5 Sec	1 cycle
	Denaturation	94°C	60 Sec	
Step II	Annealing	59°C	30 Sec	30 cycles
Step III	Extension	72°C	30 Sec	1 cycle
	Final Extension	72°C	5 min	
Hold at 4°C				

III. Agarose Gel Electrophoresis

With sample, std. 100bp ladder was loaded in different lane and run was done on 1.5% agarose gel (prepared by adding 5µl of 1mg/ml Ethidium Bromide) with 1X TAE buffer. The gel is run at constant voltage 90-95V. The resultant gel was observed under UV-trans-illuminator and compared for the PCR result.

IV. Quantification and Quality Check of DNA

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.

Quantification of DNA Sample

1. The UV spectrophotometer was switched on and the deuterium lamp was allowed to warm-up.

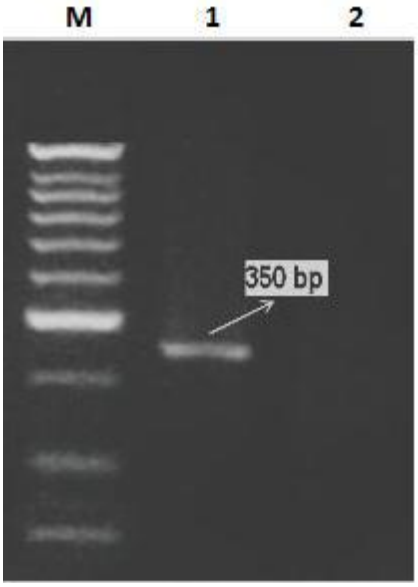
2. A blank was set with TE buffer (or distilled water) in quartz cuvettes.

3. A 1:50 dilution was prepared with isolated genomic DNA sample in distilled water and then transferred in quartz cuvettes.

4. The reading at 260 nm gives the concentration of nucleic acid in the sample while reading at 280 nm specifies the concentration of aromatic amines.

Formula OD 260nm/ OD 280nm = Ratio to determine the concentration of DNA in the sample. **DNA concentration in mg/ml = OD260 x 50 mg DNA/ml x Dilution Factor/1000** 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 quantification of amount of nucleic acid will not be possible in such a sample and it cannot be used for further processes.

Observations



LEGEND
2% Agarose Gel stained with EtBr (10mg/ml)
Lane M: 100 bp DNA Ladder
Lane 2: PCR product.

RESULTS AND DISCUSSION

PCR was run for the template DNA sample/test sample and a relatively intense band was seen around 100bp when compared with std. 100bp ladder.

The results obtained for the Bcl-2 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 that 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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