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Research Article

EFFECT OF FLUORIDATION ON CASPASE-3 PROTEIN FORMING GENE IN SWISS ALBINO MICE

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ABSTRACT
Water fluoridation is considered by many to be a pillar of public health. For several decades, fluoride has been added to water in efforts to prevent tooth decay. According to the American Dental Association, adding sodium fluoride to water supplies helps to prevent and reverse tooth decay by 20 to 40 percent. This in many areas is accepted as fact, and to not fluoridate water would be an obstruction to public health. However, many are rethinking the safety and success of such measures. In India state of Assam was reported to be affected by excessive fluoride levels in groundwater in June 2000 and many people have been crippled for life. The victims suffer from severe anaemia, stiff joints, painful and restricted movement, mottled teeth and kidney failure. Perhaps before any definite decisions are made regarding fluoride, we should investigate what pros and cons exist with water fluoridation. It has been accepted that fluorides chromosomal aberrations and gene mutations in cultured mammalian cells. Fluoride cause apoptosis and apoptotic events are thought to be mediated by the Caspase family of proteases which cleave death substrates so Caspase-3 was examine by PCR technique.

Keywords: *Fluoridation, Chromosomal Aberrations, Gene Mutations, Apoptosis, Caspase-3*

INTRODUCTION
Fluoride exists fairly abundantly in the earth's crust and can enter groundwater by natural processes; the soil at the foot of mountains is particularly likely to be high in fluoride from the weathering and leaching of bedrock with high fluoride content. The most common inorganic fluorides are HF, CaF_2, NaF, SF_6 and Silicofluorides.
Fluoride was first used to fight dental cavities in the 1940s on the basis of two grounds: 1) It inhibits enzymes that breed acid-producing oral bacteria which leach the enamel and 2) Its ions bind with calcium ions, strengthening tooth enamel as it forms in children (UNICEF).
Fluoridation to water is regarded as a simple measure to improve public health. It is thought that adding fluoride helps strengthen the enamel of teeth, preventing decay, cavities and tooth loss. This measure was endorsed by the American Medical Association in 1951 and by the American Dental Association in 1953 (Coffel, 1992).
The Clean Water Act of 1974 and subsequently amended gives the Environmental Protection Agency the authority to regulate drinking water standards, including the addition of fluoride. The FDA does not have this authority, although they have approved fluoride supplements and tooth pastes as being safe and efficacious in reducing dental decay.
According to 1984 guidelines published by the World Health Organization (WHO), fluoride is an effective agent for preventing dental caries if taken in 'optimal' amounts. But a single 'optimal' level for daily intake cannot be agreed because the nutritional status of individuals, which varies greatly, influences the rate at which fluoride is absorbed by the body. A diet poor in calcium, for example, increases the body's retention of fluoride.
Throughout many parts of the world, high concentrations of fluoride occurring naturally in groundwater and coal have caused widespread fluorosis - a serious bone disease - among local populations (UNICEF).
Nearly 100,000 villagers in the remote Karbi Anglong district in the north-eastern state of Assam were reported to be affected by excessive fluoride levels in groundwater in June 2000. Many people have been crippled for life. The victims suffer from severe anaemia, stiff joints, painful and restricted movement, mottled teeth and kidney failure. Local authorities launched a scheme for the supply of fluoride-free water

and painted polluted tube-wells red: they also put up notice boards warning people not to drink the water from these wells. (Times of India / UNI, 2 Jun 2000). Overall, Water fluoridation is not practiced in India (Ingram, 2006). UNICEF has worked closely with the Government and other partners in defluoridation programmes in India, where excessive fluoride has been known for many years to exist in much of the nation's groundwater.

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). Cohen (1997) and Kumar (1995) also commented on key role of Caspase 3 in the mechanism of apoptosis. Caspase-3 cleaves one of its substrates, poly (ADP ribose) polymerase to generate the 85 kDa fragment during apoptosis in many eukaryotic cells. The cleavage of PARP is associated with apoptotic DNA fragmentation (Ogata *et al.,* 1998).

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. A study was done to investigate whether fluorinated water can cause any mutation in Caspase-3 gene in the treated mice. The sequencing of PCR products of the gene Caspase-3 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 were 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.	CASPASE-3	Forward primer: 5′-ACG GGT CAT TTA TGG GACA-3′ Reverse primer: 5′-TAC ACG GGA TCT GTT TCT TTG-3′	422 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:

Reagents	Volume for 25µl of reaction mixture (µl)
Autoclaved distilled water	15
10X Taq DNA polymerase Buffer ($MgCl_2$)	2.5
2mMdNTPs	3
Forward primers	1.5
Reverse primers	1.5
Taq DNA polymerase	0.5
DNA Template	1
Total reaction mixture volume	**25**

2. Carried out the amplification using the above mentioned conditions and performed at least 30 cycles for amplification of the DNA fragment.

PCR Cycling Conditions for CASPASE Gene

3. 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	96°C	60 Sec	1 cycle
	Denaturation	96°C	60 Sec	
Step II	Annealing	54°C	60 Sec	38 cycles
Step III	Extension	72°C	120 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.

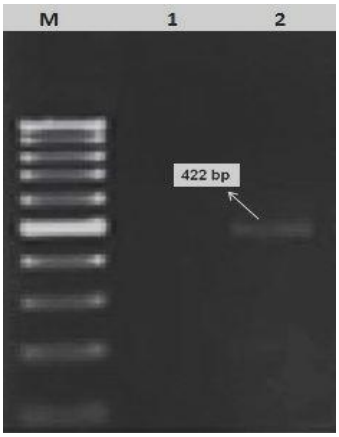
Quantification of DNA Sample

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 Caspase-3 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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