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RESEARCH ARTICLE

CREATION OF NOVEL RESTRICTION SITES *IN VIVO* IN CHICK EMBRYO NEURON-DNA AFTER ACRYLAMIDE TREATMENT-RESTRICTION MAPING ANALYSIS FOR Bam HI AND Hind III RESTRICTION ENDONUCLEASES

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Abstract

The genotoxicity of acrylamide (AC) with a significantly high chemical activity can cause many adverse effects to health of an organism. The present study investigation has analyzed the effect of acrylamide on DNA modification to insert new fragmentation sites on prolonged administration of cytotoxicity. The spectrophotometric quantification of DNA after increased administration of acrylamide to neurons shows that the amounts of DNA decrease in neurons. To test the insertion of new sites on DNA by acrylamide DNA fragment having 13.2kb length was electroeluted from the agarose gel from control and treated, and digested with *Hind III* and *Bam HI* restriction enzymes to generates restriction maps on agarose electrophoresis, and the restriction maps were analyzed by comparing with control. The acrylamide treatment has shown (0.5 and 0.6mg) significantly induced DNA damage through the formation of additional restriction site for the *Bam HI* at 3^{1} end of the neuron-DNA. From the results of the present study it was observed that the digestion of the DNA fragments from control and treated have produced unique banding pattern for each sample. It showed that the degree of the DNA damage was dose dependent
.

Introduction

The DNA damage is a relatively common event in the life of a cell and may lead to mutation, cancer and cellular death (Sancar *et al*., 2004). The genome is inherently unstable due to spontaneous chemical reactions, and its fidelity is compromised due to very low but significant replication errors (Hedge *et al*., 2008). Moreover, the genomes of all organisms are continuously exposed to a wide variety of insults, responsible for the lesions that arise in DNA. The most common insults are environmental agents, such as the ultraviolet (UV) component of sunlight, ionizing radiation and numerous genotoxic chemicals. Products of normal cellular metabolism that include ROS (superoxide anions, hydroxyl radicals and hydrogen peroxide) derived from oxidative respiration and products of lipid peroxidation cannot be forgotten. Finally, under physiological conditions, some chemical bond in DNA tends to spontaneously break, leading non-instructive abasic sites from hydrolysis of nucleotide residues (Hoeijmakers *et al*., 2001). The DNA damage if not repaired, can lead to gene mutations and genomic instability, which in turn may cause malignant transformation of cells. The individual response to DNA damage induced by xenobiotics depends largely on the efficiency of DNA repair mechanisms. Normal function of DNA repair enzymes is essential for the removal of damage. It has been shown that reduced DNA repair is associated with increased risk of cancer.

Nutrition, a most important environmental factor, plays a crucial role in short- and long-term health care outcomes. Growing evidence exists that some ingredients of preserved foods can induce epigenetic phenomena both in genotoxic and nongenotoxic ways, with long-term effects on health. Conversely, the short-term effects of non ideal food components can cause damage to cells and organs due their direct toxicities on functional systems

(Koletzko *et al.,* 2010). Among all the genotoxic and non-genotoxic pathways have been suggested more for the carcinogenic effect of acrylamide. Of the possible nongenotoxic mechanisms, acrylamide alters the redox status of cells and thus interfere on gene transcription or hormonal balance (Besaratinia and Pfeifer, 2007).

These findings on DNA damage provide us with idea that acrylamide can lead to neuronal cell death, for resulting in abnormal neuronal development or neurodegeneration (Bohr *et al.,* 2007, Nakabeppu *et al.,* 2007, Rass *et al.,* 2007). DNA lesions are known to be induced by high aerobic metabolic activities and low anti-oxidative activities in nervous system (Venkataswamy *et al*., 2013), thereby neuronal DNAs are easily damaged. These DNA lesions include are single and double strand break (SSB, DSB) on DNAs (Rass *et al.,* 2007). These breaks on DNA may occur due to acrylamide modification of bases and insertion of new nucleotides for reorganization of other restriction enzymes. However, a number of fundamental questions remained unresolved. Expression profiles of these factors in developing and mature brain are unknown. Therefore the aim of present study to investigate and analyze the effect of acrylamide on DNA damage after isolation and fractionation of neuron nucleic acid.

Material and Methods

The chemicals purchased from indigenous companies were of pure and used for the analysis in our research.

Source of fertilized eggs and incubation conditions

Freshly laid *Bobcock strain* zero day old fertilized eggs were purchased from Sri Venkateswara Veterinary University, Tirupati and Sri Balaji hatcheries, Chittoor, Andhra Pradesh. The eggs were incubated horizontally at 37.5±0.5˚C with a relative humidity of 65% in an egg incubator, we consider day1 (d1) as an incubation period of 24h. The egg incubation and collection of tissues were made based on the experimental condition laid by Venkataswamy *et al.,* 2013. The embryos were treated with 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6mg of acrylamide as single dose on day8 (d8), day9 (d9) and day10 (d10) of incubation. On 11th day the embryo's brain was collected and stored for further use at -20°C.

Isolation of neuron-DNA

Neuronal enriched fractions from chick cerebral cortex were prepared essentially as standardized earlier (Usha rani *et al.,* 1983). The neuronal cell rich fraction was washed with PBS and homogenized in lysis buffer. After centrifugation for 5min at 1600Xg the supernatant was collected and the extraction procedure was repeated with the same amount of lysis buffer.

The supernatants (and the nuclei as control for the complete recovery of the apoptoic DNA fragments) were brought to 1% SDS and treated for 2h with RNase-A (final concentration 5µg/ml) at 56^0C followed by digestion with Proteinase-K (final concentration 2.5µg/ml) for at least 2h at 27^0C. After addition of half volume of ammonium acetate the DNA was precipitated with 2.5 volumes of absolute ethanol and centrifuged at 1600Xg. Alcohol was drained by inverting the tubes on blotting paper then the pellet was dissolved in TE buffer, p^H-8.4.

Qualitative and quantitative analysis of DNA by nano microlitre spectrophotometer

Analysis of UV absorption of nucleotides provides a simple and accurate estimation of the concentration of nucleic acids in a sample. The DNA absorption was measured at 260nm and DNA content was recorded using the calculation as, concentration of DNA (µg/ml) =OD260 × dilution factor. A ratio 1.8-2.0 denotes that the absorption in the UV range is due to pure nucleic acids. Ratios lower than the 1.8 indicates the presence of proteins and or other UV absorbants (Lakshmi Narasaiah *et al.,* 2012). A ratio higher than 2.0 indicates that the samples may be contaminated with chloroform or phenol.

Separation of DNA by Agarose gel Electrophoresis

Neuronal genomic DNA (7µl) was fractionated on 0.9% agarose gel electrophoresis (Sambrook *et al*., 1989); about 15µg of the sample with 5µl of the loading dye and marker DNA were loaded carefully in to the respective wells without disturbing the gel. After conducting the electrophoresis at 50mV, agarose gel was photographed using gel doc.

Fragmentation by restriction enzymes

Analysis of DNA molecules is often hampered by their large size. One way of generating specific smaller DNA molecules from larger ones is through the use of restriction enzymes, which are sequence specific DNA endonucleases. The two restriction enzymes we used to generate DNA fragments were:

Bam HI - from Bacillus amyloliquefaciens site which cut in between G&G of - 5' G G A T C C 3'; and Hind III - from Hemophilus influenzae site which cut in between A&G of - 5' A A G C T T 3' to produce cohesive ends.

Each group was set up using one of the restriction enzymes, in a 0.5ml microcentrifuge tube. The frozen DNA samples were thawed slowly and kept on ice into series of eppendrof tubes. 1.2µl of RE buffer, 2µl of 1mg/ml of BSA, 5µl of DNA samples and 200µg/ml RNase were transferred, and all tubes were made up to 20µl of final volume with 11.5µl of milli Q water and 0.3µl of restriction enzyme was added to all tubes and incubated for 2h at 37^0C. After Incubation, the tubes were kept in 70^o C water bath for 10min to inactivate restriction enzymes. Stored restriction digests at -20^o C as 4µl aliquots and each reaction mixture was mixed with 16µl of loading buffer and analyzed by agarose gel electrophoresis. After electrophoresis run, the gels were photographed by observing the transilluminated DNA fragments on UV transilluminator.

RESULTS

Determination of DNA in chick embryonic neurons

The DNA has been isolated from Control and acrylamide treated chick embryonic neurons by the procedure of Herrmann and Kalden (1994) were determined by calculating the ratio of absorbance between 260 and 280nm (Warburg and Christian, 1941). The content of DNA was 3.918µg/µl of control neuron samples (**Figure-1**). The initial concentration of acrylamide 0.1mg has showed 3691, 3684, and 3566ng/µl of DNA with the interval of 24, 48 and 72h, and 5.79, 5.95, and 5.97% of variation of decrease in levels were found compared to control. In 0.2 mg of AC treated embryo the content of DNA were 3446, 3191, and 3133ng/µl, and 12.05, 18.55, and 18.91% of variation DNA decreased levels when compared to control. At 0.3mg of AC treated embryos content of DNA 2851, 2516, and 2130ng/µl and 27.23, 35.78 and 45.01% of variation DNA decreased levels when compared to control. At 0.4 mg of AC treated embryos content of DNA was 1935, 1789, and 1653ng/µl and 50.61, 54.33, and 57.33% of variation DNA decreased levels when compared to control. At 0.5mg of AC treated embryos content of DNA was 1604, 1024, and 929ng/µl and 59.06, 73.86, and 76.01% of variation DNA decreased levels when compared to control. At 0.6mg of AC treated embryos content of DNA was 727, 606.3, and 606.3ng/µl, and 81.44, 84.53, and 84.55% of variation DNA decreased levels when compared to control (**Figure-1**). The average decreased percentage of variation observed for 0.1mg to 0.6mg acrylamide treatment of chick embryo neurons were 5.90, 16.50, 36.0, 54.09, 69.64, and 83.50. The 50% of variation was observed at 0.4mg acrylamide treatment in between the timings of 48 and 72h. However the degree of variation found was more at first three doses (0.1 to 0.3) than the remaining higher three doses (0.4 to 0.6)

Fractionation of DNA

The isolated genomic DNA of control and treated were fractionated on 0.9% agarose gel electrophoresis (Sambrook *et al*., 1989) as mentioned in materials and methods. Agarose gel photograph has showed that the degree of percent variations of DNA was increased as the concentration of acrylamide increased. The **Figure-2** showed a comparative damaged DNA fragment patterns of control and acrylamide treated brain neuron-DNA. The lane-2 is the control DNA sample treated with saline. Lane-3-6 (0.1-0.4mg) acrylamide treated groups showed marginal DNA damage at lower concentrations (i.e. 0.1, 0.2, 0.3, 0.4mg, of AC) and at higher concentrations lane-5&6 (0.5 and 0.6mg of AC) marked DNA damage at 13.2kb region (**Figure-2**).

Restriction digestion analysis

In the present study a major DNA fragment, 13.2kb (**Figure-2**), of electrophoressed gel was electroeluted for both controls and AC treated, and were digested with restriction endonucleases, *Bam HI* and *Hind III*, separately, and their DNA trailing patterns were analyzed by agarose gel electrophoresis using Gene marker DNA ladder mix (Fermentas-USA) as a marker. The **Figures-3** has shown a comparative restriction fragment patterns for controls and acrylamide treated neuron-DNA digested with *Hind III* enzyme with two bands at 8.4 and 4.8kb. The **Figures-4** has shown a comparative restriction fragment patterns of controls and acrylamide treated neuron-DNA digested with *Bam HI* enzyme with the observation of three bands at 5.2, 4.6 and 3.4kb. The restriction patterns observed with this enzyme were unique to each other and marked differences were observed mainly at 0.5 and 0.6mg AC treated and the trailing of DNA patterns are increased as the concentration of AC increased. The **Figures-5** has shown a comparative restriction fragment patterns of controls and acrylamide treated neuron-DNA digested with *Hind III* and *Bam HI* enzyme with the observation of four bands at 4.6, 3.8, 3.2, and 1.4kb at Lane-2-6, treated with 0.1 to 0.4mg acrylamide and five bands (extra band) at 2.6, 1.4 and 0.9kb (at Lane-7 and Lane-8) along with Lane-2-6 bands, treated with 0.5 and 0.6mg acrylamide.

Figure-1: Effect of acrylamide on neuron-DNA levels

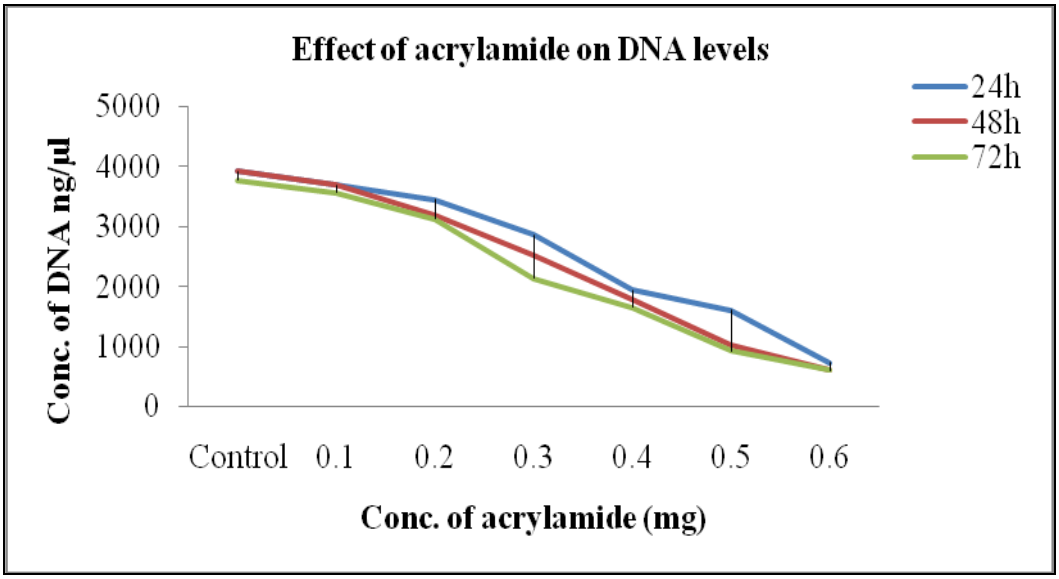
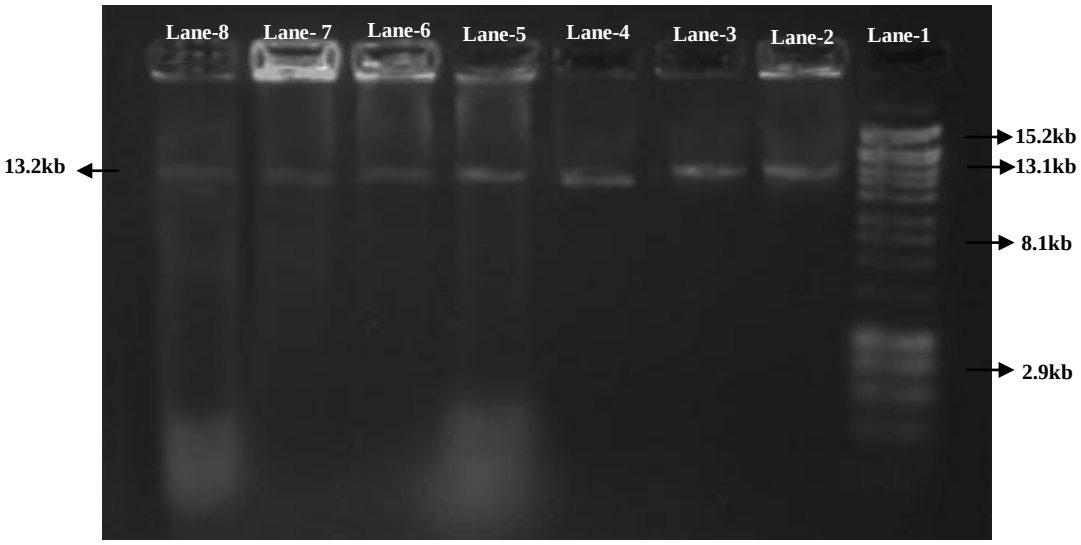
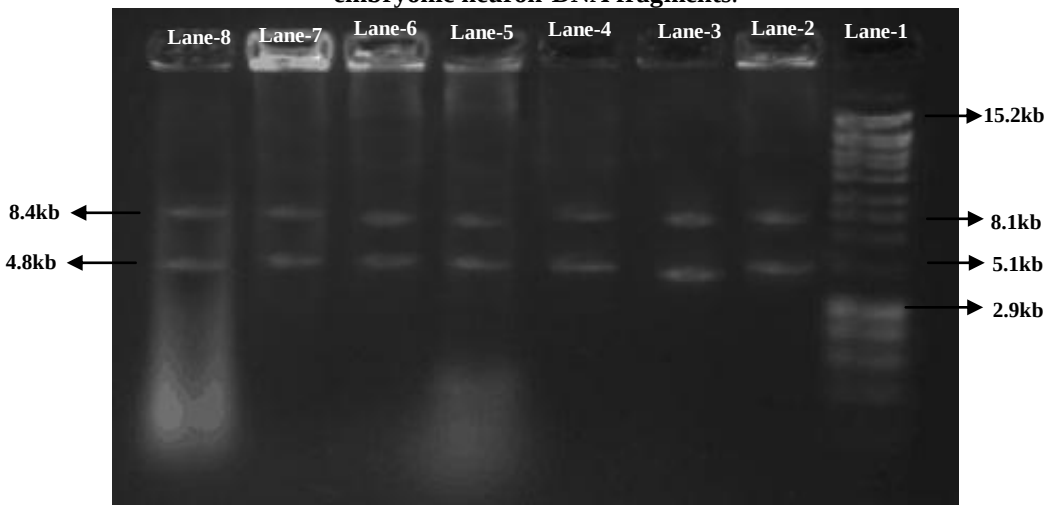


Figure-2: Electrophoresis analysis of control and treated chick embryo neuron-DNA (13.2kb)



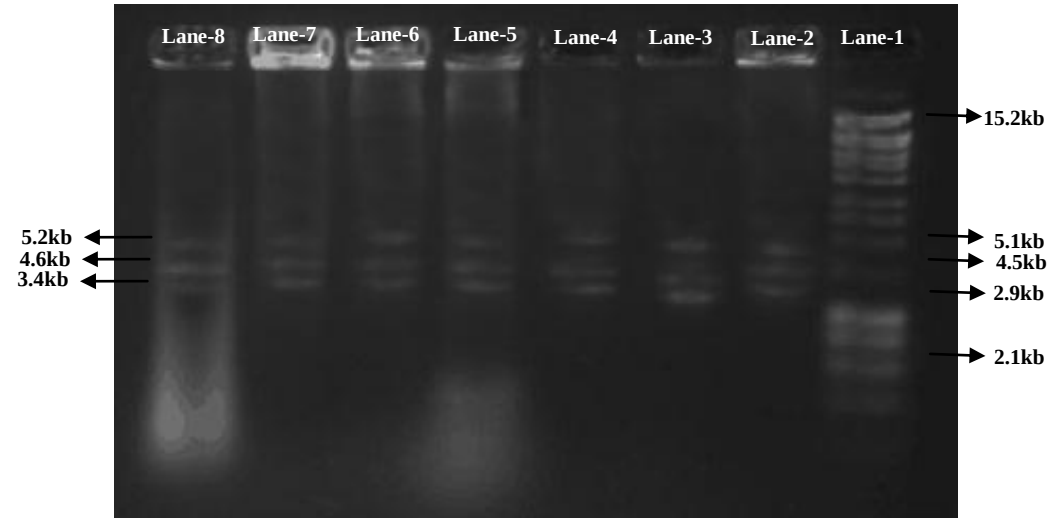
Lane-1: Marker (gene rular DNA Ladder mix [Fermentas-USA]), **Lane-2**: Control, **Lane-3**: 0.1mg AC, **Lane-4**: 0.2mg AC, **Lane-5**: 0.3mg AC, **Lane-6**: 0.4mg AC, **Lane-7**: 0.5mg AC, and **Lane-8**: 0.6mg AC treated brain neuron-DNA.

Figure-3: Hind III restriction digestion analysis of electro eluted control and acrylamide treated chick embryonic neuron-DNA fragments.



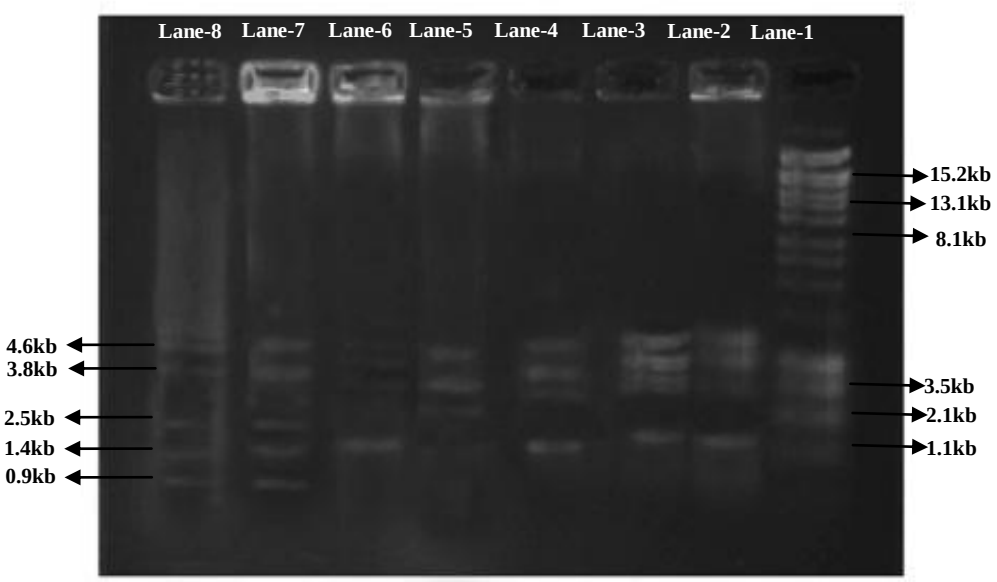
Lane-1: Marker (gene rular DNA Ladder mix [Fermentas-USA]), **Lane-2**: Control, **Lane-3**: 0.1mg AC, **Lane-4**: 0.2mg AC, **Lane-5**: 0.3mg AC, **Lane-6**: 0.4mg AC, **Lane-7**: 0.5mg AC, and **Lane-8**: 0.6mg AC treated brain neuron DNA.

Figure-4: Bam HI restriction digestion analysis of electro eluted control and acrylamide treated neuron-DNA fragments.



Lane-1: Marker (gene rular DNA Ladder mix [Fermentas-USA]), **Lane-2**: Control, **Lane-3**: 0.1mg AC, **Lane-4**: 0.2mg AC, **Lane-5**: 0.3mg AC, **Lane-6**: 0.4mg AC, **Lane-7**: 0.5mg AC, and **Lane-8**: 0.6mg AC treated brain neuron DNA.

Figure-5: Hind III and Bam HI restriction digestion analysis and electro eluted control and treated neuron-DNA fragment (13.2kb).



Lane-1: Marker (gene ruler DNA Ladder mix [Fermentas-USA]), **Lane-2**: Control, **Lane-3**: 0.1mg AC, **Lane-4**: 0.2mg AC, **Lane-5**: 0.3mg AC, **Lane-6**: 0.4mg AC, **Lane-7**: 0.5mg AC, and **Lane-8**: 0.6mg AC treated brain neuron-DNA.

Figure-6: Restriction Map of developing chick embryonic neuron-DNA (13.2kb)

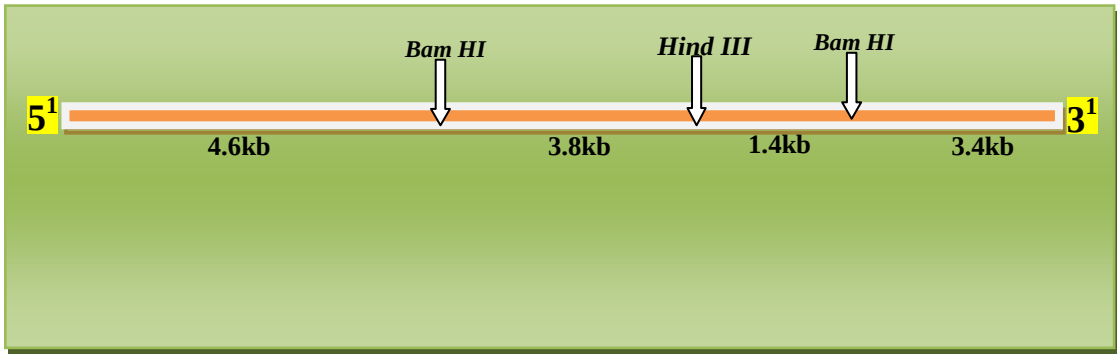
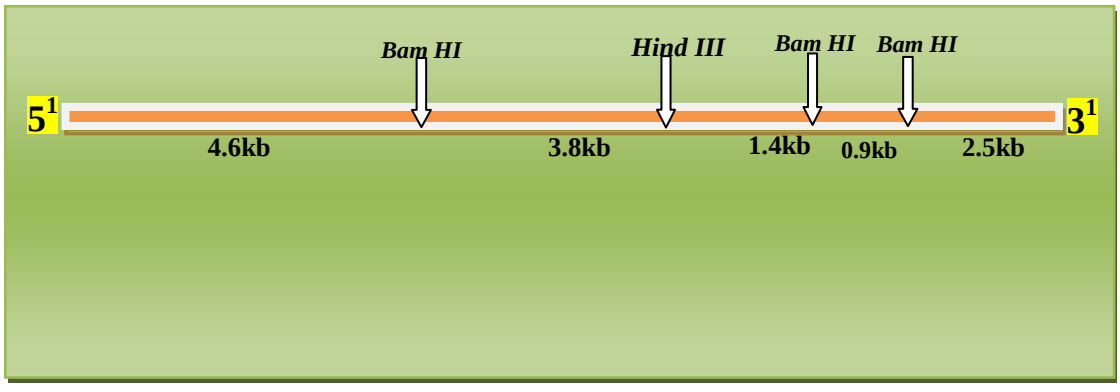


Figure-7: High doses of acrylamide treated (0.5 and 0.6mg AC) restriction Map of developing chick embryonic neuron-DNA (13.2kb)



Discussion

The identification of cellular DNA damage targets of exogenous reactive acrylamide has garnered significant attention in an attempt to understand the genotoxic and cytotoxic mechanisms of this molecule. The endogenous products of acrylamide reaction with cellular macromolecules have been suggested to participate in diseases associated with oxidative stress. In addition to forming adducts to protein and DNA, acrylamide induces Schiff base-mediated cross-link between DNA and protein. Daniel *et al*., 2005 have previously characterized a depurinating DNA adduct, N7-(2-carbamoyl-2-hydroxyethyl) guanine (N7-GA-Gua), from the *in vitro* reaction of DNA with GA and detected this adduct in rodent tissues following a single intra peritoneal injection of (^{14}C)-substituted AC. Thus, AC may represent a mode for DNA-protein cross-link formation *in vivo*. Although a mechanistic link between DNA-protein cross-links and disease has not been elucidated, the formation of cross-links can be detrimental to cell survival. The bulky nature of the cross-links is likely to disrupt DNA metabolism by posing significant blocks to replication and transcription. The acrylamide intake by the embryonic cell has revealed the decrease in the content of DNA due to its concentration increase. The percentage of variation of DNA depletion was more with high doses (0.5 to 0.6mg) and increased time intervals (48 to 72h). Based on spectrophotometric analysis our result of acrylamide influence on chick embryo neurons has concluded that the decrease in DNA content due to dose and time dependent features. However, the influence of acrylamide on DNA at initial levels was more (**Figure-1**).

The present study has demonstrated the ability of AC to cause DNA damage *in vivo* by the restriction endonucleases digestion analysis, *Bam HI* and *Hind III*. The neuron-DNA of developing chick embryonic restriction map observed in **Figure-6**. One important question at present is the impact of AC in food to the cancer risk in humans. But, despite the fact that AC is a known carcinogen in rodents, the underlying mechanism of action is not fully understood. AC has been reported to possess clastogenic and mutagenic properties in vivo. However, AC itself is known to react quite slowly with DNA (Nicole *et al.*, 2005). Therefore, its metabolite GA, formed by CYP2E1, is proposed to represent the ultimate carcinogen (Sumner *et al.*, 1999). AC and its metabolite GA are known to be clastogenic and mutagenic *in vitro* and *in vivo* (Paulsson *et al*., 2003 and Costa *et al.*, 2003). From the restriction digestion analysis the selected restriction fragment 13.2kb has shown a slight variable polymorphism by depicting its length from 13.2 to 13.0kb. At high doses of acrylamide treatment (0.5and 0.6mg AC) induced mutations and forms the additional restriction site for the *Bam HI* at 3^1 end of the neuron-DNA (**Figure-7**).

In accordance with these data, we found substantial genotoxicity in all two cell types tested (Blood cells and germ cells) with highly significant induction of DNA strand breaks in the range of 0.4mg AC treatment. Only at the highest concentration (0.5 and 0.6mg AC) DNA strand breaks were induced, without substantial differences in sensitivity between these cells different species origin. The present data showed the DNA damage at higher concentrations i.e. above 0.5-0.6mg of AC treatment. At high acrylamide treated, the concentration of DNA is very low and it shows that the high degree of apoptosis in neurons and it supported by high degree of variations in restriction digestion of electroeluted apoptotic DNA with *Bam HI* and *Hind III* analysis. AC treated groups when compared with control have showed that the concentration of apoptotic DNA is increased as the concentration of AC increase and it is augmented at the range of 0.6mg of AC as a threefold decrease. The restriction digestion analysis of 13.2kb range fragments of electroeluted apoptotic DNA of treated groups with *Bam HI* and *Hind III* have also supported with its high degree of variation in their gel patterns.

The changes in DNA caused by genotoxic chemicals may be monitored using different biomarker assays both at biochemical and molecular level (Savva, 1998). The RFLP profiles detect alterations in genomic DNA with the use of arbitrarily restriction digestion reactions and clearly show in the detection of acrylamide-induced DNA effects. However, it is only a qualitative method through which nature and amount of DNA can only be speculated. The present study shows the first report on acrylamide induced RFLP profiling in developing chick embryonic neuron. The changes observed in the DNA profiles such as modifications in band intensity and loss of bands may be due to the changes in oligonucleotide restriction sites mainly due to genomic rearrangements, and less likely to point mutations or DNA damage in the binding sites or the presence of DNA photoproducts which can block or reduce the polymerization of DNA in the PCR reaction (Nelson *et al*., 1996). Appearance of bands could be attributed to the presence of oligonucleotide restriction sites which become accessible to oligonucleotide restriction digestion or after structural change or because some changes in DNA sequence have occurred due to mutations (Atienzar *et al*., 1999) (resulting in new annealing events) or large deletions (bringing two pre-existing annealing sites closer) or homologous recombination.

CONCLUSIONS

The research findings of acrylamide on neuron DNA conforms that the acrylamide induces modification of DNA for generation of new sites for degradation of DNA. This may further causes mental disorders in animal systems due to creation of variable DNA sites

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