Genotoxicity Evaluation of acute doses of arsenic trioxide and Chromium nitrate to fresh water teleost *Channapunctatus* (Bloch.) by alkaline single cell gel electrophoresis(comet assay).

RakhiChaudhary[a], K.K.Gaur[a], B.Kushwaha[b], N.S. Nagpure[b], RavindraKumar[b].

[a]*R.B.S. College, Agra, U.P. (India).*

[b]*National Bureau of Fish Genetic Resources, Lucknow, U.P. (India).*

Email-*rakhi.r.singh@gmail.com*

Abstract :

Arsenic and chromium are major environmental pollutant and exposure occurs through environmental, occupational and medicinal sources. The contaminated aquatic system is the main source of exposure and affects the aquatic fauna and ultimately the human beings. The present investigation aimed to study the induction of DNA damage by arsenic trioxide and chromium nitrate in fresh water teleost fish Channapunctatus using micronucleus assay (MN assay) and alkaline single cell gel electrophoresis (comet assay). The value of LC50-96h of arsenic trioxide and chromium nitrate was determined for C.punctatusin a static system and on the basis of LC50 value four concentrations 5%, 10%, 20% and 40% of L.C50 value were determined. The fishes were exposed to the different concentrations and samplings were done at regular intervals for genotoxicity assessment. It was found that micronucleus inductions increased from first to last day while tail DNA increases upto fourteenth day and then decreased. More DNA damage reported in arsenic trioxide exposed fishes as compared to chromium nitrate.This investigation explored the utility of genotoxic biomarkers for determining the potential of various environmental toxicants.

Keywords : ***Comet assay, DNA damage, arsenic trioxide, fish, chromium nitrate, genotoxicity.***

INTRODUCTION :

Arsenic and chromium are widely used heavy metals and bioaccumulative in fishes and can be indirectly harmful to human populations. Arsenic and its compounds are known for their high toxicity. Exposure sources are food, air, water, occupational settings and medicines. Recently, large population in West Bengal in INdia and Bangladesh has reported to be affected and also other countries like China, Taiwan, Thailand, Chili, Romania affected. (Smith et al. 2000; GuhaMazumder et al. 1998). World health organization (WHO) and US environmental protection agency (EPA) had set up the standard for drinking water known as MCL which is 10 µg/l.

Chromium (Cr) is one of the least toxic metals on the basis of itsover supply and essentiality. According to recent estimate, in India, 2000-3200 tones of elemental chromium escape into the environment annually from the tanning industries alone (Thiagrajan, 1992).

Commonly chromium used in Dyeing, pigmenting, textile and as a refrectory materials. Chromium concentration in Tannery effluent usually ranges between 2000-5000 μg ml^{-1} Chromium pollution occurs due to indoctrinate discharge of untreated tannery water. The total wastewater let out from the tanneries in the country has been estimated to be 3008-3324 liters/100 kg of hides processed. Chromium compounds are known to have toxic, genotoxic, mutagenic and carcinogenic effects on man and animals (Von Burg and Liu, 1993; Stoks and Bagchi, 1995; Mont and Hockett, 2000), through various routes above two heavy metals reaches to aquatic environment and tend to accumulate in the tissues of aquatic organisms and can be indirectly harmful to human populations.

The present study deals with the effect of arsenic trioxide and chromium nitrate on fresh water fish Channapunctatus using comet assay in blood and gill tissue.

Materials and method :

For the present study practical grade Arsenic trioxide and Chromium nitrate (manufactured by CDH laboratory) was procured from the local market.

Experimental fish Specimens :

The healthy specimens of the freshwater fish Channapunctatus (Bloch.), (family :Channidae and order : Cypriniformes), were procured from local sources. The average wet weight and length (± S.D.) of specimens were 20 ± 3g and 10 ± 3 cm, respectively. The specimens were treated with 0.05% $KMnO_4$ solution to avoid any dermal infections. Fishes were then acclimatized in laboratory conditions for two weeks in semi-static systems prior to exposure to arsenic trioxide and chromium nitrate. Fishes were fed with boiled eggs. The foecal matter and other waste materials were siphoned off daily to reduce the ammonia content in water. Every effort, as suggested by Bennett and Dooley (19820, was made to maintain optional conditions during acclimatization.

Determination of LC_{50}values :

The acute toxicity bioassays for determination of 96 hr. LC_{50} values of arsenic trioxide and chromium nitrate were conducted in the static system. The acute bioassay procedure was based on standard methods (APHA-AWWA-WPCF, 1998). The stock solution of arsenic trioxide was prepared by dissolving in HCl. While chromium nitrate easily soluble in water. A set of 10 fish specimens were randomly exposed to each chemical and the experiment was repeated twice for obtaining the 96 hr. LC_{50} value of lest chemicals for the species.

The LC_{50} values of arsenic trioxide and chromium nitrate was determined as 98.33 mg/L and 740 mg/L respectively following the probit analysis method as described by Finney (1971).

In vivo exposure experiment :

The fish specimens were exposed to LC_{50} values of arsenic trioxide and chromium nitrate in static system. Mortality was reported during experimentation. Four specimens were withdrawn from each test chemical at 1^{st}, 3^{rd} and 7^{th} day for collection of blood and gill tissue. The fish maintained in tap water and fish specimens exposed to HCl were considered as control and vehicle control, respectively. On each sampling day, th whole blood and gills were collected and immediately processed for comet assay. The blood samples were collected from the fish by heart

puncture technique using heparinized syringe. The physico-chemical properties of test water namely temperature, pH, conductivity, total alkalinity, and dissolved oxygen were analyzed by standard methods (APHA-AWWA-WPCF, 1998).

Single cell gel electrophoresis or comet assay :

Immediately after collection the blood samples were initially mixed with PBS and then the comet assay protocol was followed as described by Singh et. al. (1988) with slight modifications as standardized in our laboratory for this fish species. Gill tissue after collection were washed three times with chilled PBS (Ca^{++}, Mg^{++} free) to remove most of the red blood cells. For preparation of comet slides 15 µl cell suspension (approx. 20,000 cells) were mixed with 85 µl of 0.5% low melting point agarose and layered on one end frosted plain glass slide, previously coated with a layer of 200 µl normal agarose (1%). The agarose cell sandwich was covered with the third layer of 100 µl low melting point agarose (0.5%). After solidification, the slides were immersed in lysing solution (2.5 M NaCl, 100 mM na_2-EDTA, 10mM Tris, pH .10 with 10% DMSo, and 1% Triton X-100 added fresh) for overnight at 4°C. After lysis, slides were placed side by side in a horizontal electrophoresis unit containing fresh, cold alkalie electrophoresis buffer (300 m MNaOH, 1 mM Na_2-EDTA and 0.2% DMSO, pH 13.5) and left in the solution for 15 min at 4°C for DNA unwinding and conversion of alkali0labile sites to single strand breaks. Alkaline electrophoresis was performed by using the same buffer for 10 min using 16V at 4°C. The slides were then washed thrice for 5 min. with neutralization buffer (0.4 M Tris, pH 7.5) to remove the excess alkali. The slides were stained with ethidium bromide (20 µg/ml, 5 min) or visualization of the DNA damage for positive control, the whole blood were treated ex vivo with 100 µM H_2O_2 for 10 min. at 4°C. Two slides per specimen were prepared and 200 cells per concentration (25 cells per slide) were scored randomly using an image analysis system ('Komet-5'; Kinetic Imaging) attached to a fluorescence microscope equipped with the appropriate filters. The percent DNA (= 100^- percent Head, DNA), as determined by the software, was taken as the parameter of DNA damage.

Statistical Analysis :

Two way ANOVA with replication was applied to compare the mean differences in the % Tail DNA among blood and gill, concentrations and time period.

Results :

Physico-chemical properties of test water :

The temperature and pH of water ranged from 25 to 27°C and 7.5 to 8, respectively. The dissolved oxygen content was normal and varied from 7.5-8.5 mg/L during the experimental period. The conductivity and total alkaliity of the test water ranged from 253 to 310 µM Cm^{-1} and 280 to 325 mg/l as $CaCO_3$ respectively.

DNA damage :

The fish specimens, exposed to acute concentration of arsenic trioxide and chromium nitrate, exhibitred significantly higher DNA damage in their tissues as compared to the control, whereas the damage in vehicle control was non-significantly higher than control. A significant effect of duration of exposure has been observed in the fish specimens exposed to arsenic

trioxide. The highest DNA damage was observed in blood on seventh day (32.51%). The tail increases from first day to seventh day. Further in gill tissues also tail progressed from first day to seventh day. The tail increased from 20.55% to 30.84%.

The chromium nitrate showed less toxic than arsenic trioxide. In blood DNA damage ranges 12.72% to 20.89% from first day to seventh day. Gill tissues showed maximum damage on first day 22.7% and then decreased on seventh day 15.85%.

Discussion :

The comet assay under alkaline conditions (pH > 13) (Singh et. al. 1988) is able to detect DNA damage, i.e. single strand breakage or other lesions such as alkali-labile sites, DNA cross-links (Tice, 1995) and incomplete excision repair events, (Gedik et. al. 1992). It offers considerable advantages over the other cytogenetic methods like chromosome aberrations, sister chromatid exchanges an micronucleus test used to detect DNA damage, because for comet assay the cells need not to be mitotically active (Buschini et. al., 2003). Therefore, it has been widely used in the fields of genetic toxicology and environmental biomonitoring (Tice, 1995) including aquatic organisms (MItchelmore and Chipman, 1998; Lee and Steinert, 2003) as a powerful tool for measuring the relationship between DNA damage and the exposure of aquatic organism to genotoxic pollutants.

The genotoxicity studies can be important approach to have greater insight into the organism's ability for DNA repair and other protective mechanisms to excrete the toxic chemicals. Further, the tissue specific responses to the particular chemical can also be explored. In case of fishes, use of crythrocytes for the evaluation of DNA damage using the comet assay (Pandrangi et. al.; 1995; Belpaeme et. al. 1996, 1998; Mitcholmore and Chipman, 1998; Sumathi et. al. 2001) seems to be advantageous due to the ease of tissue collection and processing for comet assay especially in case of smaller fishes.

Arsenic and chromium have been found to be carcinogenic to human and/or animals. However the mechanism involved in their carcinogenicity are still unclear (Goering et. al. 1999). Indirect genotoxic effects of metal salts have been discussed eq. the generation of oxidative DNA damage, and the interference with DNA repair and DNA replication processes (Dally et. al. 1999, Hartwig et. al. 1998; Hartwig et. al. 1997). DNA lesios induced by metals can consist of DNA single and/or double strand breaks. DNA-DNA cross links, DNA-protein cross-links and base modifications.

Cells treated with arsenic trioxide showed a significant dose-dependent increment in the extent of DNA migration and the percentage of "tailed" cells. These results were in agreement with those found by Hartmann (1994) and (1996). OUr results also agree with those reported by Von Burg and Liu (1993), Blasiak and Kowalik (2000) and Matsumoto et. al. (20030, who proposed that chromium exerts a genotoxic effect on animals due to its potential to cause various forms of DNA damage. The DNA damage in the gill cells could be explained by the gill being the most appropriate target organ that is directly and constantly exposed to the DNA – damaging chemicals dissolved in the water (Dzwonkowska and Hubner, 1986). The suitability of gill tissues for genotoxic studies has also been demonstrated earlier using shellfish (Sasaki et. al., 1997). The observed tissue-specific responses may also be due to physiological activities distinctive to these

organs, with respect to either the activation or detoxification of particular pollutants or the repair of the different types of strand breaks.

The genotoxic potential of acute doses of both heavy metals showed dangers to aquatic fauna. However, further studies are needed to explore DNA damage in aquatic organisms after heavy metals exposure so that right action for abatement can be carried out.

CONCLUSION :

Arsenic trioxide and chromium nitrate found to be acutely toxic in *C. punctatus* exposed to it in static system. Further, the presence of DNA strand breakage in exposed specimens indicated the genotoxic potential of heavy metals.

It can also be concluded on the basis of our results that the comet assay showed high senstivity in detection of DNA damage in blood and gill tissues of fish. Therefore, the comet assay can be successfully employed for in vivo DNA damage studies using fish as model species for environmental biomonitoring studies.

ACKNOWLEDGEMENT: Author highly obliged to R.B.S.COLLEGE ,AGRA and NATIONAL BUREAU OF FISH GENETIC RESOURCES ,LUCKNOW for giving permission and support for conducting research work.

References :

A. Hartmann, G. Speit. Comparative investigations of the genotoxic effects of metals in the single cell gel (SCG) assay and the sister chromatid exchange (SCE) test, Environ. Mol. Mutagen. 23 (1994) 299-305.

APHA, AWWA and WPCF, (1998).Standard methods for the Examination of water and wastewater, 20th Ed. Am. Publ. Hlth. Assoc., New York.

Belpaeme, K., Cooreman, K., KIrsch-Volders, M. (1998).Development and validation of the in vivo alkaline comet assay or detecting genomic damage in marine flatfish.mutat. Res. 415, 167-184.

Belpaeme, K., Delbeke, K., Zhu, L., KIrch-rolders, M., (1996). Cytogenetic studies of Comet assay. Mutagenesis 11, 485-492.

Benett, R.O. and J.K. Dooley, (1982).Copper uptake by two sympatric species of Killifish Fundulusheteroclitus (L) and l. Majalis (Walkbawn). J. Fish. Biol. 21(4), 381-398.

Buschini, A., Carboni, P., Martino, A., POli, P., Rossi, C., (2003). Effects of temperature on baseline and genotoxicant-induced DNA damage in haemocytes of Dreissenapolymorpha, Mutat. Res. 537, 81-92.

C. Abernathy, Y.Liu, D. Longfellow, H. Aposhian, B. Beck, B. Fowler, R. Goyer, R. Menzer, T. Rossman, C. Thompson, M. Waalkes, Arsenic, health effects, mechanisms of action and research issues. Environ. HEalthPerspect. 107 (1999) 593-597.

Dzwonkowska, A., Hubner, H., 1986. Induction of chromosomal aberrations in the syrian hamster by insecticides tested in vivo. Arch. Toxicol. 58, 152-156.

Finney, D.J., (1971). Profit Analysis. University Press, Cambridge, P. 333.

Gedik, C.M., Ewen, S.W.B., Collins, A.R., (1992). Single cell gel electrophoresis applied to analysis of UV-C damage and it's repair in human cells. Int. J. Radiat. Biol. 62, 313-320.

GuhaMazumder, D.. U.C. Ghoshal, J. Saha, A. Santra, B.K. De, A. Chatterjee, S. Dutta, C.R. Angle and J.A. Centeno: Roundomized Placebo controlled trial of 2, 3-dimercapto succinic acid in therapy of chronic arsenicosis due to drinking arsenic contaminated subsoil water. Clin.Toxicol, 36, 683-690 (1998).

H. Yan, C. Carter, C. Xu, P. Singh, M. Jones, J. Johnson, M. Dietrich. Cadmium induced apoptosis in the urogenital organs of the male rat and its suppression by chelation, J. Toxicol. Environ. Health 52 (1997) 149-168.

Lee, R.F., Steinert, S., (2003).Use of single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and fresh water) animals.Mutat. Res. 544, 43-64.

Mitchelmore C.L. and Chipman J.K. (1998). DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. Mutat. Res. 399, 135-147.

Mount DR and HOckett JR (2000) Use of toxicity identification evaluation methods to characterize; identify, and confirm heravalent chromium toxicity in an industrial effluent. Water Res. 34: 1379-1385.

P. Goering, H. Aposhian, M. Mass, M. Cebrian, B. Beck, M. Waalkes. The enigma of arsenic carcinogenesis: role of metabolism. Toxicol. Sci. 49(1999) 5-14.

Pandraugi, R., Petras, M., Ralph, S., and Vrzoc, M., (1995). Alkaline single cell gel (comet) assay and Genotoxicity Monitoring, using Bullheads and Corp. Environmental and Molecular Mutagenesis 26, 345-356.

Sasaki, Y.F., Nishidate, E., ishibashi, S., Tsuda, S., Matsusaka, n., Asano, N., Saotome, K., Sofuni, T., Hayashi, M., 1997. Detection of genotoxicity of polluted sea water using shellfish and alkaline single cell-gel electrophoresis (SCE) assay: a preliminary study. Mutat. Res. 393, 133-139.

Singh, N.P., Mc Coy, M.T., Tice, R.R.l and Schneider, E.L., (1988). A simple techynique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 1841-91.

Smith, A.H., A.P. Arrays, D.N. GuhaMazumder, M.J. Komett, A.L. Hermadez, M. Beeris, M.M. Smith and L.E. Moore: Arsenic induced skin lesions among atacamens people in NOrthern Chile despite good nutrition and centuries of exposure. Environ. Hlth. Perspect, 108, 617-620 (2000).

Stohs SJ and Bagchi D. (1995) Oxidative mechanisms in the toxicity of metal ions.Free Radic Biol. med. 18: 321-336.

Sumathi M., Kalaisehi K., Palanivel M., Rajaguru P., (20010. Genotoxicity of textile dye effluent on fish (Cyprinuscarpio) measured using the comet assay. Bull. Environ. Contam.Toxicol. 66, 407-414.

T. Hei, S. Liu, C. Waldren, Mutagenicity of arsenic in mammalian cells: role of reactive oxygen species. Proc. Natl. acad. Sci. U.S.A. 95 (1998) 8103-8107.

Thiagragan, g. (1992). In pursuit of better image : survey of Environment. The hindu, 143-145.

Tice, R.R., (1995). The single cell gel/comet assay a microgel electrophoresis technique for the detection of DNA damage and repair in individual cells. In philips DH. Venitt S. editors.Environmental Mutagenesis. Oxford: Bios Scientific Publishers, 315-339.

Von Burg R and Liu D. (19930.Chromium and hexavalent chromium. J. Appl. toxicol. 13: 225-230.

W. Dally, A. Hartwig, Induction and repair inhibition of oxidative DNA damage by nickel (II) and cadmium (II) in mammalian cells, Carcinogenesis 18 (1997) 1021-1026.