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INDIRECT SAFETY ASSESSMENT OF ECTODERMAL MERCURY EXPOSURE BY TRADITIONAL MEDICAL FORMULATIONS ON FRESHWATER CAT FISH *CLARIAS BATRACHUS* USING MICRONUCLEUS ASSAY AND ALKALINE SINGLE-CELL GEL ELECTROPHORESIS (COMET) ASSAY

Anand Prem Rajan*, Nathiya T and Alphonse Maria A
School of Bio Sciences and Technology, VIT University, Vellore, India

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***Corresponding author**
Email: aprdbt@gmail.com

ABSTRACT
Mercury and its salts are the major constituents of Ayurvedic, Chinese and Tibetan traditional formulations. The mercury is extensively reported to accumulate in food chain and cause many neurological disorders in environment. The safety assessment of mercury on the ectodermal application on animal model is rarely reported. The void in the scientific study on external exposure of mercury and its genotoxic inside the body lead to the necessity for the present study. The freshwater cat fish *Clarias batrachus* was used for broad specificity genotoxic indicators micronucleus assay and alkaline single-cell gel electrophoresis (comet) assays. The fish was exposed to 0.03 ppm of mercuric chloride for a period of 7, 14, 28 and 35 days ectodermally. The blood sample was assayed for the genotoxicity. The results revealed undoubted DNA damage through the micronuclei and alkaline single-cell gel electrophoresis (comet) assays. Hence it is concluded the usage of traditional medicines containing the mercury may be toxic at genetic level in prolonged usage.
Key words: Mercury, traditional medicines, micronucleus assay, alkaline single-cell gel electrophoresis (comet) assay, *Clarias batrachus*

INTRODUCTION

Worldwide pursuit for the examination of traditional medical system is gearing up in the recent years. The Indian ayurvedic system and herbal medicines have attracted the global attention due to their slow and explicit cure[1,2].

Mercury is indigenously used in Indian system of ayurvedic medicine e.g. Bhasm (plant parts mixed with mercury and fixed with heat) is used as medicine. In China and Tibet, mercury use was thought to prolong life, heal fractures, and maintain generally good health. Mercury chloride has traditionally been used as diuretic, topical disinfectant and laxative. Mercury in the form of one of its common ores, cinnabar, remains an important component of Chinese, Tibetan, and Ayurvedic medicine. The effects of the mercury are well documented which proposes prohibition of export of these medicines. Methyl mercury compounds are possibly carcinogenic to humans. However, it is still unclear about the extent of period which induces neuron damage, and dosage which inhibits brains development and maturation[3]. Both acute and chronic exposure produces permanent changes to affected organs and organ systems[4]. Acute poisoning due to mercury vapors affects the lung primarily, in the form of interstitial pnuemonitis, bronchitis, and bronchiolitis[5].

The traditional experimental methods may not reveal the insight of the chromosomal and genetic effects of a chemical agent. Hence elite genotoxics studies are required to access adverse effects of any chemical substance. Micronuclei (MNi) assay is most widely applied method due to its proven suitability for fish species[6]. The micronucleus test detects both clastogenic and aneugenic effects and therefore can detect the genotoxicity of a wide range compounds. Increased frequency of micronuclei in fish cells have been shown under both field and laboratory conditions after the exposure of cells to different genotoxic chemicals and their complex mixtures[6]. Micronuclei are cytoplasmic chromatin masses, with the appearance of small nuclei that arise from chromosome fragments or intact whole chromosome lagging behind in the anaphase stage of cell division. The presence in cells is a reflection of structural and/or numerical chromosomal aberrations arising during mitosis. Micronucleus formation as well as induction of nuclear alterations was considered to be the consequences of genotoxic events in fish[7].

The Single Cell Gel Electrophoresis is an uncomplicated and sensitive technique for the detection of DNA damage at the level of the individual eukaryotic cell[8]. The comet assay under alkaline conditions (pH > 13) is able to detect DNA damage, i.e. single strand breakage or other lesions, such as alkali–labile sites, DNA cross-links[9] and incomplete excision repair even[10].

DNA lesions have been detected by induced chemical mutagens using the single-cell gel electrophoresis. The alkaline SCGE assay was extensively used to detected genotoxicity of chemical in gill cells of shellfish[11]. The DNA damage between tissues is explained by the number of the alkali–labile sites, being variable in DNA from different tissues and by the different cell types having different background levels of DNA single strand breaks due to variation in excision repair activity, metabolic activity, antioxidant concentrations and other factors. It has since gained in popularity as a standard technique for evaluation of DNA damage/repair, biomonitoring and genotoxicity testing. The use of biological test system for monitoring Ayurvedic and traditional medicines is gaining importance worldwide.

Fishes are generally used as toxicity indicators. Exothermic vertebrates, teleosts (bony fish), a large phylogenetic group of fish including over 20,000 extant species exhibit specific in vivo as well as in vitro immune response to various antigenic stimuli. Recently mercury chloride induced changes in the DNA, RNA, protein and alkaline phosphatase of kidney of fresh water teleost *Labeo nandina*[12]. Many authors recommend fish as the

best animal model for toxicological studies[13,14]. *Clarias batrachus*, which belongs to an air breathing catfish species, found primarily in Southeast Asia is robust species as it "walk" across dry land, to find food or suitable environments, was used as the test organism in the present study.

The effects of ectodermal mercury exposure by different fungicidal and traditional formulations are not found during the extensive literature survey. The present study was undertaken to fill this scientific void using high tech cellular and molecular techniques using the fish *Clarias batrachus* as model organism.

MATERIAL AND METHODS

Animal

Healthy specimens of *Clarias batrachus* were procured from Ranipettai. Fish of same age and size which hatched from the same lot of eggs (broodstock) were collected; Fish were transported to the Environmental Biotechnology laboratory for acclimatization and experiment was conducted in CO_2 & Green Technology Center of VIT University.

Acclimatization

Acclimatization was done by stocking fishes in a large, rectangular cement tank (4m x 6m x 3m), previously soap washed, disinfected with potassium permanganate and thoroughly rinsed thrice prior to filling with water.

Fish were acclimatized to laboratory condition for a fortnight, before being used for experiments. No symptoms of disease were apparent and no antibiotics or other medical preparation were utilized for disease acclimatization and also during subsequent periods. During acclimatization, the stock was maintained at natural photoperiod and ambient temperature and fed ad libitum once daily with groundnut oil cake and rice bran, both being powdered in the ratio of 1:2 and given in the form of dough. Water was replaced for every 24 hr and well aerated in order to reduce any accumulation of excretory products and ensure sufficient oxygen supply to fish. Feeding was withheld for 48 hours prior to the commencement of all experiments so as to keep the experimental animals in same metabolic condition and to minimize possible effects on measured parameters during experiments.

Experimental Setup

The glass-aquaria of 50 L capacity which were cleaned, previously disinfected with potassium permanganate, sun dried and filled with clean water were used for genotoxical studies. 0.03 ppm i.e. $1/10^{th}$ of 24h LC_{50} concentration of Analytical grade mercuric chloride (Fisher Inorganic and Aromatics Limited, Madras, India) was used in the present study[15]. A control (50 fish) without mercury chloride was maintained simultaneously. Experiment was conducted for 35 days. 5 fishes were randomly selected from control and experimental aquaria at weekly intervals i.e., (7, 14, 21, 28 and 35 days) and blood was collected for the genotoxicological studies without being anesthetized for analysis.

Collection of blood

Blood was drawn from the caudal vein by using plastic disposable syringe fitted with 26 gauge needle which was already moisture with heparin, (Beparine, heparin sodium, IP 2000 IU ml^{-1}, derived from intestinal mucosa containing 0.15 percentage w/v cholesterol IP preservative) an anticoagulant manufactured by Biological E limited, Hyderabad, India. Blood collected from treatment and control was expelled into the separate heparinized plastic vials and placed immediately on ice. Pooled blood sample was used for determination of all the parameters[15].

Genotoxicological Studies

Micronuclei test

Two drop of blood sample obtained from catfish was smeared on glass slide. The slide was then fixed with methanol for 15 minutes and air dried. The next day it was stained with 15% giemsa solution for 10 minutes. The slide was dehydrated in alcohol and cleaned in xylene. Slides were mounted in DPX for the microscopic observations[16].

Single cell gel Electrophoresis (Comet assay)

The glass slide was dipped in 1% normal melting agar for first layer and allowed for setting for 5 min at 4°C. Fish blood (containing cells) was added to 80µl of 0.65% low melting agar in PBS (Dissolve the following in 800ml distilled H_2O.8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4, 0.24g of KH_2PO_4, Adjust pH to 7.4. Adjust volume to 1L with additional distilled H_2O. Sterilize by autoclaving). Transfer this to the slide for producing the final layer. After solidification of agar the slide was placed in the cold lyses solution (2.5M NaCl, 100mM EDTA, 10mM Tris-HCl, pH 10, 1% sodium sarcosinate, 1%Triton X-100, 10% DMSO, pH 10) at 4°C overnight in dark. The slide were removed gently and placed in horizontal gel box containing electrophoresis buffer (1mM Na2EDTA, 300mM NaOH, Adjust pH to 13) for 20 minutes. The electrophoresis was performed in ice water bath. After electrophoresis the slide was washed three times in neutralization buffer (Tris-HCl buffer pH 7.5). Then the slide was photographed using gel documentation[17].

Statistical Analysis

The statistical analysis was made individually on each sample and the mean value of five individual observations was taken for each parameter.

RESULT

The results of the present study showed that the mercury is potential even when body is exposed externally by medications containing mercury.

Micronuclei

The thorough examination of the blood smears showed the evidences of micronuclei in treated fish (Fig 1).

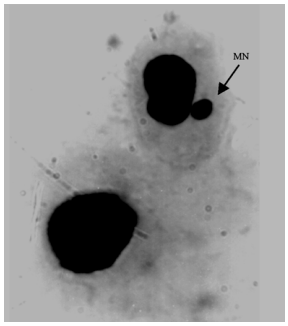

Fig. 1: Blood cell of *Clarias batrachus* exposed to mercuric chloride. The micronuclei (MNi) is shown by an arrow

Single cell gel electrophoresis (SCGE)

The Single Cell Gel Electrophoresis of the fish blood revealed significant DNA damage (Fig 2). The extent of damage extended with the exposure period. The comet image was not witnessed in control fish blood. Results show that the number of DNA damaged cells is proportional to exposed pollutant. Table 1 showing the number of Micronuclei enumerated during the experimental period of 35 days.

Table 1: Number of Micronuclei enumerated during the experimental period of 35 days

Days Exposed	Control	Treatment
7	1	10
14	1	15
28	2	19
35	3	25

DISCUSSION

Heavy metals natural product of Indian Ayurvedic medicine and traditional medicines used throughout the world[18]. The traditional medicine is consumed for a longer period of time unlike allopathic medicines. The mercury present in the medicine is in extremely small quantities. The results of the present study on ectodermal exposure of Mercuric chloride showed that both geotoxicity indicators MNi and SCGE were positive and were time dependent. Detection of DNA strand breakage is a relatively sensitive, rapid and broad specificity indicator of genotoxic pollutant exposure[19]. The results are coherent with the studies on redbreast sunfish (*Lepomis auritus*) where SCGE that increased the level of single breakage in hepatic DNA isolated from toxic loaded waters[20].

DNA damage may be due to the breakage of single or double strand breaks which may occur due to various mechanisms[21]. The strand breakage may also cause due to various excision repair enzyme [22] Strand breaks may also be produced via alkali-liable sites. Therefore measurement of single strand breakage can be used to analyze genotoxic effect of some chemicals. The presence of single strand break fragments of DNA to move from the nucleoid core towards the anode, thus resulting in 'comet' formation[22]. More damage results in increase in tail length due to more migration of DNA fragments. The comet assay has been described as being very sensitive (detecting 1 break described in 1 x 10^{10} Da[23].

CONCLUSION

The traditional medicines containing mercury may be effective against the fungi and other microorganism but continuous, prolonged and extensive use may causes similar genotoxic in humans. The sophisticated molecular tests like MNi and SCGE supports these facts beyond doubt.

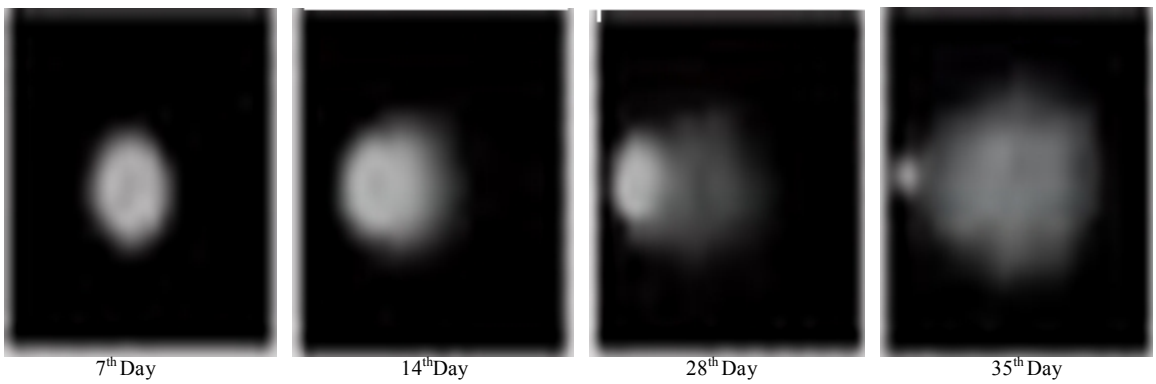

Fig 2: Shows the picture of Single Cell Gel Electrophoresis of cat fish (*Clarias batrachus*) blood in weekly intervals

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