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Open Access Journal
International Journal of Medical Research and Pharmaceutical Sciences
Volume 4 (Issue 7): July 2017 ISSN: 2394-9414
DOI- 10.5281/zenodo.831536 Impact Factor- 3.109

INVESTIGATION OF CARCINOGENIC AND MUTAGENIC PROPERTY OF FOOD COLOR USING CAT FISH CLARIAS BATRACHUS BY USING ALKALINE SINGLE-CELL GEL ELECTROPHORESIS (COMET) ASSAY AND MICRONUCLEUS ASSAY

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Keywords: *Allura Red AC (E129), micronucleus assay, COMET assay,Clarias batrachus*

Abstract

In recent years, humans are exposed to various carcinogenic agents, artificial colors like Green #3, Red#3, Citrus red #2, Saccharin, Etc. The present study emphasis is made to know the effect of these common agents in particular with reference to artificial food color Allura red (FD & C # 40)- orange red food dye on DNA damage using Cat Fish *Clarias batrachus* as an model organism. The methodology will be to understand direct damage to nucleus by Micronuclei test and Chromosomal damage by alkaline single-cell gel electrophoresis (COMET) ASSAY. The freshwater fish catfish *Clarias batrachus* was used for specificity genotoxic indicators micronucleus assay and COMET assays. The fish was exposed to 1g/L of Allura red (FD & C # 40) for a period of 7, 14, 28, and 35 days ectodermally. The blood sample was tested for genotoxicity. The results revealed DNA damage through alkaline single-cell gel electrophoresis (comet) and micronuclei assays. Hence it is concluded that the usage of food color containing Allura red(FD & C # 40) –orange red food dye may be toxic at genetic level if the usage is prolonged

Introduction

Food was fresh and natural in the early ages. Due to population explosion there was a demand for increase in productivity of consumer goods which made an impact on producers to add more chemical compounds (food additives) to increase the level of yield and to make it look more fresh and good. Azodyes, amaranth, Allura red and coccine which are currently used as food color additives in Japan, have been reported to cause colon specific DNA damage in mice[16]. Food additives show various symptoms of threats on long term and short term exposures.

So both acute and chronic exposures produce permanent changes to affected organs[2]. Hence genotoxic studies are needed to study the effect of any chemical compound or substance. Micronuclei test is the most widely used assay due to its proven correctness for fish species[4]. Micronuclei presence in cells is due to the structural and numerical chromosomal aberrations arising during mitosis.

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The Single Cell Gel Electrophoresis is a responsive technique for the detection of DNA damage at the level of individual cell[6]. The comet assay under alkaline conditions (pH>13) can detect DNA damage, i.e. single strand breakage or DNA cross-links and incomplete excision repair even[7]. DNA lesions have been detected by inducing chemical mutagens using Comet assay.

Fishes are generally used as toxicity indicators. Teleosts and many exothermic invertebrates show immune response to antigenic stimuli. Many researchers suggested fish as the best animal model for these studies[8, 9]. *Clarias batrachus* belongs to the air breathing cat fish species, found in Southeast Asia is robust since it can walk in search of food across dry land. This is the species which is being used as our test organism.

The present study is made to know the effect of these common agents in particular with reference to artificial food color Allura red (FD & C # 40) - orange red food dye on DNA damage using Cat Fish *Clarias batrachus* as model organism.

Materials And Methods

Animal

Healthy specimens of *Clarius batrachus* were bought from Ranipet. Fishes of same age and size were collected and were transported to the Environmental Biotechnology laboratory for acclimatization, after which experiment was conducted in CO_2& Green Technology Center of VIT University[1].

Acclimatization

Acclimatization was done by stocking fishes in a rectangular cement tank (5m x 7m x 4m), which was initially washed with soap, disinfected with potassium permanganate and thoroughly rinsed thrice former to filling with water. Fish were acclimatized to laboratory condition for a night, before being used for experiments. No symptoms of disease were noticed during succeeding periods. During adaptation, the stock was maintained at normal temperature and fed once daily with fish food (In the form of balls). Water was regularly changed for every 24 hours and well aerated in order to reduce any accumulation of excretory products and to make sure whether there is sufficient oxygen supply to fish. Feeding was stopped for 48 hours before the experiments were started so as to keep the experimental animals in same metabolic condition[1].

Experimental setup

The glass-aquaria of 70L capacity which were cleaned, was filled with clean water were used for genotoxic studies. 1g/L i.e. 1g of Allura red (FD & C # 40)– orange red food dye was dissolved in 1 liter of water and was used in the present study. A control (20 fish) without Allura red AC (E129) was maintained simultaneously. Experiment was conducted for 35 days. 5 fishes were randomly selected from control and experimental aquaria at regular intervals (weekly) i.e. (7, 14, 21, 28, and 35 days) and blood was collected for genotoxicological studies.

Collection of blood

Blood was drawn from the caudal vein by using plastic disposable syringe fitted with 22 gauge needle which was already moisture with heparin,(Beparin, heparin sodium, IP 2000 IU ml^{-1}, derived from intestinal mucosa containing 0.15 percentage w/v cholesterol IP preservative) an anticoagulant. 2.5ml of blood was expelled from the caudal vein. The blood collected from treatment and control was expelled into the separate heparinized centrifuge tubes and was placed immediately in the refrigerated condition. This blood sample was used for determination of all the parameters[1].

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Genotoxicological Studies

Micronuclei test

Two drop of blood sample was taken from catfish and a smear was made 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 and then the slide was dehydrated in alcohol and cleaned in xylene. Slides were mounted in DPX for observing under the microscope[1].

Single cell gel electrophoresis (Comet assay)

The glass slide was dipped in 1% normal melting agar for first layer and allowed forgetting set for 5min 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 water. NaCl -8g, Na_2HPO_4 - 1.44g, KCl-0.2g,KH_2PO_4 - 0.24g, pH was adjusted to 7.4. Volume was adjusted to 1L with addition of distilled water. Sterilize by autoclaving). This was transferred to the slide to produce the final layer. After solidification of agar the slide was kept in the cold lyses solution (2.5M of NaCl, 100mM of EDTA, 10mM of Tris-HCl, pH-10, 1% of sodium sarcosinate, 1% of Triton X-100, 10% of DMSO, pH 10) at 4°C overnight in dark. The slide were taken gently and placed in horizontal gel box containing electrophoresis buffer (1mM of Na_2EDTA, 300mM of NaOH, and adjust pH to 13) for about 20 minutes. The electrophoresis was performed in cold water bath. After electrophoresis the slide was washed three times in neutralization buffer (Tris-HCl buffer of pH 7.5). Then the slide was being photographed using gel documentation[1].

Statistical Analysis

The statistical analysis was made individually on each sample and the mean values of five individual observations were taken for each observation.

Result

The results of the present study showed that the food color Allura Red (FD&C Red #40) - orange / red food dye is even potential enough to induce cancer on a long term exposure.

Micronuclei test

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

Fig 1. Micronuclei test result of the exposed test organism Cat fish Clarias batrachus

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Single cell gel electrophoresis

The test showed remarkable results as there was significant DNA damage in the blood cells. The level of damage was differing from week to week based on the time of exposure. The pictures below depict the level of damage in regular time intervals.

Discussion

Fish serve as useful genetic models for the evaluation of pollution in aquatic ecosystems and hence in the present study we have used *Clarias batrachus*as the model organism. The erythrocyte micronucleus test has been used with different fish species to monitor aquatic pollutants displaying mutagenic features[17]. So the experimental fishes were tested for the micronuclei formation after the exposure time and the found results were coherent with the studies conducted with redbreast sunfish from toxic loaded waters which had increased level of single breakage of DNA when tested with SCGE[10].

Food colors or additives are widely used in edible items mostly, by manufacturers to make it look fresher and to make it more appetizing. Though it succeeds in playing its role well it causes major damages in the DNA[3]. One of such Food additives like Allura Red AC (E129) (FD&C Red #40) - orange / red dye used in food items was tested in the present study. The results of present study showed a significant DNA strand breakage and proved the above fact to be evident. Detection of DNA strand breakage is a sensitive indicator of genotoxic exposure as seen above.

DNA damage might be because of single or double stranded breakage. Therefore measurement of single strand DNA breakage can be used as one of the sensitive tests to detect genotoxic effect of some chemical compounds. COMET assay is one of the most sensitive techniques to test the DNA breakage. That is one of the confirmatory tests we have done in the present study and the basic principle behind comet assay is that the presence of single strand breaks of DNA fragments that move them from the nucleoid core towards the anode, thus resulting in 'Comet' formation[11]. It is a very sensitive test and helps in getting remarkable results in relative studies.

The micronuclei test is employed both for laboratory assays of genotoxicity of many compounds and for *in situ* surveys to assess the risk of mutagen -polluted environments[17]. In the present study it is observed that Food additives like Allura Red AC (E129) (FD&C Red #40) - orange / red dye used in food items are carcinogenic is proved with positive results in micronucleus test and single cell gel electrophoresis (COMET) assay when tested on the model organism *Clarias batrachus.*

Conclusion

Food additives like Allura Red AC (E129) (FD&C Red #40) - orange / red dye used in food items might make the food more appealing and could taste better but its exclusive property of causing DNA damage should be taken into consideration and should be banned from usage for human welfare. Since both the genotoxicological studies i.e. micronucleus test and single cell gel electrophoresis (COMET) assay had given positive results. It is advisable to reduce the intake of these kinds of food additives in our day to day lives.

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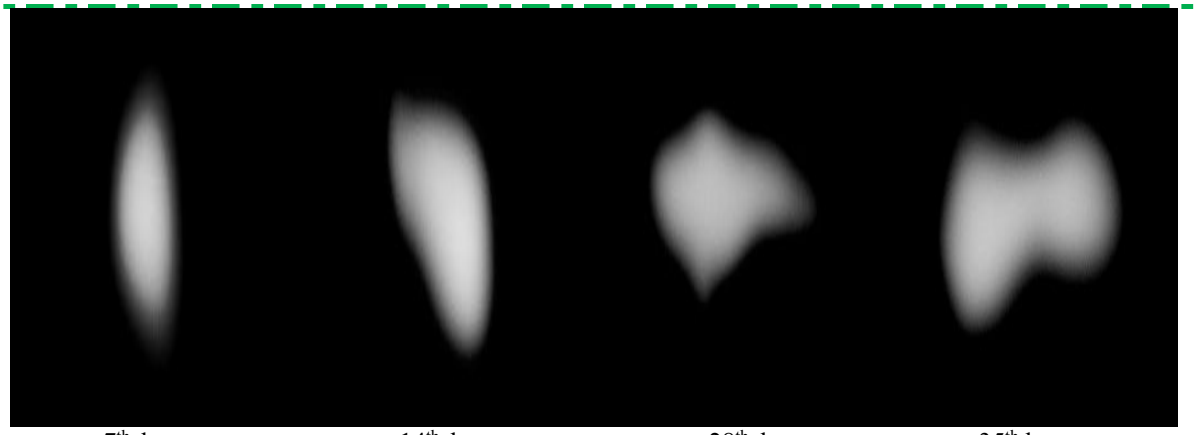

7th day 14th day 28th day 35thday

Fig 2: Pictures of single cell gel electrophoresis test taken in weekly intervals Clarias batrachus

Acknowledgements

Authors want to thank the management of VIT University for providing research facilities. Our special thanks go to The Manager, Dr. R. Natrajan, CO_2 & Green Technology Centre for supporting the studies by providing work space and manpower for fish studies as well as stock maintenance. Presently Shamundeeswari Anandan, is doing PhD in the Department of Clinical Sciences, University of Bergen, Haukeland University Hospital, Bergen, Norway. Author wants to thank All-Powerful God. Whoever does not love, does not know God, because God is love. This is how God's love was revealed among us; God send His one and only Son - Jesus Christ into the world, so that we might live through Him [Ref: 1John 4:8&9].

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