International Journal of Scientific Research in Biological Sciences **Research Paper**
Vol.8, Issue.6, pp.09-15, December (2021) **E-ISSN:** 2347-7520
DOI: https://doi.org/10.26438/ijsrbs/v8i6.915

Genotoxic impacts of long term exposure of Arsenic and Fluoride to Cat Fish, *Clarias batrachus*

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Available online at: www.isroset.org

Received: 05/Oct/2021, Accepted: 17/Nov/2021, Online: 31/Dec/2021

Abstract - Arsenic and Fluoride are very common ground water pollutants, contributed by natural and anthropogenic sources leading to serious health effects in both terrestrial and aquatic organisms. The current research was carried out to identify the relationship between arsenic and fluoride, using *Clarias batrachus* (common cat fish) as an experimental model. The study includes seven groups (Group I to VII), one control (Group I) and other six (Group II, III, IV, V, VI, VII) exposed to different concentration of arsenic and fluoride individually and in combination. Long term exposure of 60 days was carried out for each group, with no toxicant added in Group I. Blood samples were collected from each experimental group on 60th day and COMET assay was performed to check genotoxicity. Parameters like Head DNA percentage, tail DNA Percentage, comet length, tail moment were calculated from comet images. Results revealed maximum DNA damage in group V, which was exposed to arsenic alone, concluding arsenic being more toxic than fluoride. Also, antagonistic relationship was established between arsenic and fluoride.

Keywords - *Arsenic, Fluoride, Clarias batrachus, Genotoxicity*

I. INTRODUCTION

Increasing anthropogenic activities are the key to aquatic and terrestrial pollution across the globe. Arsenic and Fluoride are very common ground water pollutants, contributed by both natural and human caused reasons. Concurrent occurrence of fluoride and arsenic is widespread in many states of India, China, Bangladesh and South East Asia. Following their exposure, severe health complications arise namely Arsenicosis and Fluorosis, respectively [1].

Fluoride gets accumulated in aquatic invertebrates and vertebrates, through food and water. Once absorbed, it is distributed and accumulated in various organs and body parts [2]. The tendency of fluoride accumulation is found more in bony tissues than in soft tissues [3]. Fluoride also alters numerous hematological parameters [4] and interferes with various enzymatic (SOD, Catalase) and non- enzymatic activities (GSH, LPO) [5]. It has propensity to bind with the DNA molecule and distort its normal structure, and also induces generation of free radicals, ultimately causing DNA damage [6].

Arsenic, on the other hand is a non essential heavy metal which is mainly contributed by coal burning industries [7], generally present in its two forms organic and inorganic; inorganic being the more toxic one [8]. Aquatic organisms get exposed to arsenic through food/dietary sources and via water. Arsenic intrudes in the food chain and hence has bioaccumulative properties [9]. Accumulation of arsenic in fishes takes place in different organs like liver, kidney and gills depending upon the source of exposure [10]. Arsenobetaine, a water-soluble arsenic compound is usually found in marine living beings, which affects the organism and also can have adverse effects on humans indirectly [11]. Arsenic inhibits various enzyme activities involved in DNA repair leading to DNA damage. It brings about oxidative stress and free radicals affecting DNA and various cellular activites [6].

Contradictory literatures are available for both antagonistic and synergistic relationship of fluoride and arsenic. However, adverse health consequences of arsenic and fluoride exposure individually have been explored more, in comparison to their combination effects [12].

The research paper is further described as follows; section II mentions about the methods acquired to carry out the experiment, section III deals with the finding of the research carried out and also the discussion in which our results were supported by other researchers. Future scope and conclusions are narrated in section IV, followed by acknowledgement and finally the references.

II. RELATED WORK

Research reports are available confirming concurrent presence of arsenic and fluoride in various water bodies; but a very little work has been explored about the effects of both toxicants on aquatic organisms. However, many reports are available on rats. Arsenic and fluoride lead to increased activities of glutathione peroxidases, SOD & catalase, and also reduce levels of glutathione and ascorbic

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acid [13]. Similar results are available in ovary of rats [14] and blood, liver and brain of rats, exposed to different concentrations of arsenic and fluoride, concluding antagonistic connection between them [15]. Correspondingly, antagonistic associations were noticed between the two, wherein increased ROS and lipid peroxidation were observed in groups of rats exposed to arsenic and fluoride separately [16].

Several conflicting publications are also available explaining synergistic association of fluoride and arsenic. Reports have been published, observing less DNA damage in groups of rats exposed to both arsenic and fluoride in combination, demonstrating synergistic effects between the two [17].

III. METHODOLOGY

Experimental model, *Clarias batrachus* were selected for the study and were purchased from local market. Before acclimatization, fishes were immersed in 1% $KMnO_4$ solution, to avoid any skin diseases. Five fishes in each water tank of 50L capacity were acclimatized for 15 days with continuous supply of oxygen and water, with room temperature maintained at 27°C. They were fed daily once with Taiyo fish food.

Sodium fluoride (NaF) (Himedia laboratory) and Arsenic trioxide (As_2O_3) (Sigma Aldrich) of desired concentration were used to expose Clarias *batrachus.* Seven experimental groups were formed to study the effects of fluoride and arsenic individually and in combinations. Group I (Control), Group II (10 mgL^{-1}NaF), Group III (20 mgL^{-1}NaF), Group IV (1 mgL^{-1} As_2O_3), Group V (2 mgL^{-1} As_2O_3), Group VI (10 mgL^{-1}NaF + 1 mgL^{-1} As_2O_3), Group VII (20 mgL^{-1}NaF + 2 mgL^{-1} As_2O_3)

Long term exposure of 60 days was carried out for each group, with no toxicant added in Group I, and the desired toxicant were added in the following groups. Blood samples were collected from each experimental group on 60^{th} day. 1.0ml each of blood samples were collected in polypropylene vials/tubes coated with heparin. Samples were stored at 4◦C for about a week or at a temperature of -20°C, until further analysis.

Comet Assay:
Samples were then analyzed for DNA damage by using COMET assay technique [18], a SCGE (Single Cell Gel electrophoresis) of blood cell, which signifies the amount of DNA impairment. The procedure involves preparation of base slides, using layers of LMPA (Low Melting Point Agarose) and NMA (Normal Melting agarose). Blood samples collected were dissolved in PBS (Phosphate Buffer Saline) solution for isolation of blood cells. Samples were then placed on prepared base slide and coated with agarose layer, and cover slips gently placed over it. Microgel slides were subjected to electrophoresis for about 20 minutes (15 volts & 300 mill amperes). Slides were stained with EtBr (Ethidium bromide) and immediately visualized under fluorescent microscope (Leica DM 1000).

Visual scoring method developed by Comet assay forum (www.cometassayindia.org)was used. Around 100 cells were counted per slide & analyzed for damage. CaspLab (Comet assay Software Project 1.2.3b), was also used for calculating comet length, percentage of DNA in head and tail region in COMET images.

IV. RESULTS AN DISCUSSIONS

Results
DNA damage was analyzed using Comet assay technique in blood samples of control and experimental fishes of different concentration of NaF & As_2O_3. Least DNA damage was found in control samples with maximum number of 'Type 0' and minimum number of 'Type 4' DNA damage categories. Greatest damage was observed in group V ($2\mu gL^{-1}$As) with maximum 'Type 4' and minimum 'Type 0' (Figure 1). Less percentage of DNA damage was found to be observed in groups exposed to both fluoride and arsenic (group VI & VII), compared to groups exposed only to arsenic (group IV & V).

Highest percentage of DNA in head region was observed in control group (Figure 2). Order of Head DNA (%) witnessed was; group I (99.4±0.29) > group II > group III > group VI > group IV > group VII > group V (36.60±5.32). However, tail DNA (%) was found maximum in group V (63.39±9.22) and least in group I. Decreasing order of tail DNA was found in group V, followed by group VII, group IV, group VI, group III, group II, group I (0.56±0.82).

Highest length of the comet was found in group V (304±3.21) followed by group VII, group IV, group VI, group III, group II. And least length was seen in group I (40.4±2.20) (Figure 3)

Tail moment was also estimated, which is the product of tail DNA percent & tail length of the comet. Highest tail moment was measured in group V and lowest was detected in control group (Figure 4).

COMET images (Figure 5), using blood samples collected from fishes of each group was allowed to run in electrophoresis (SCGE) and were observed under fluorescent microscope. Maximum DNA damage, with long comets were observed in group V (arsenic treated) and least DNA damage were reported in control samples (group I). DNA damage was found be highest in arsenic exposed groups than in fluoride exposure groups. Confirming the antagonistic nature of arsenic and fluoride, DNA damage was low in groups exposed to arsenic and fluoride together as compared to groups exposed to fluoride and arsenic individually.

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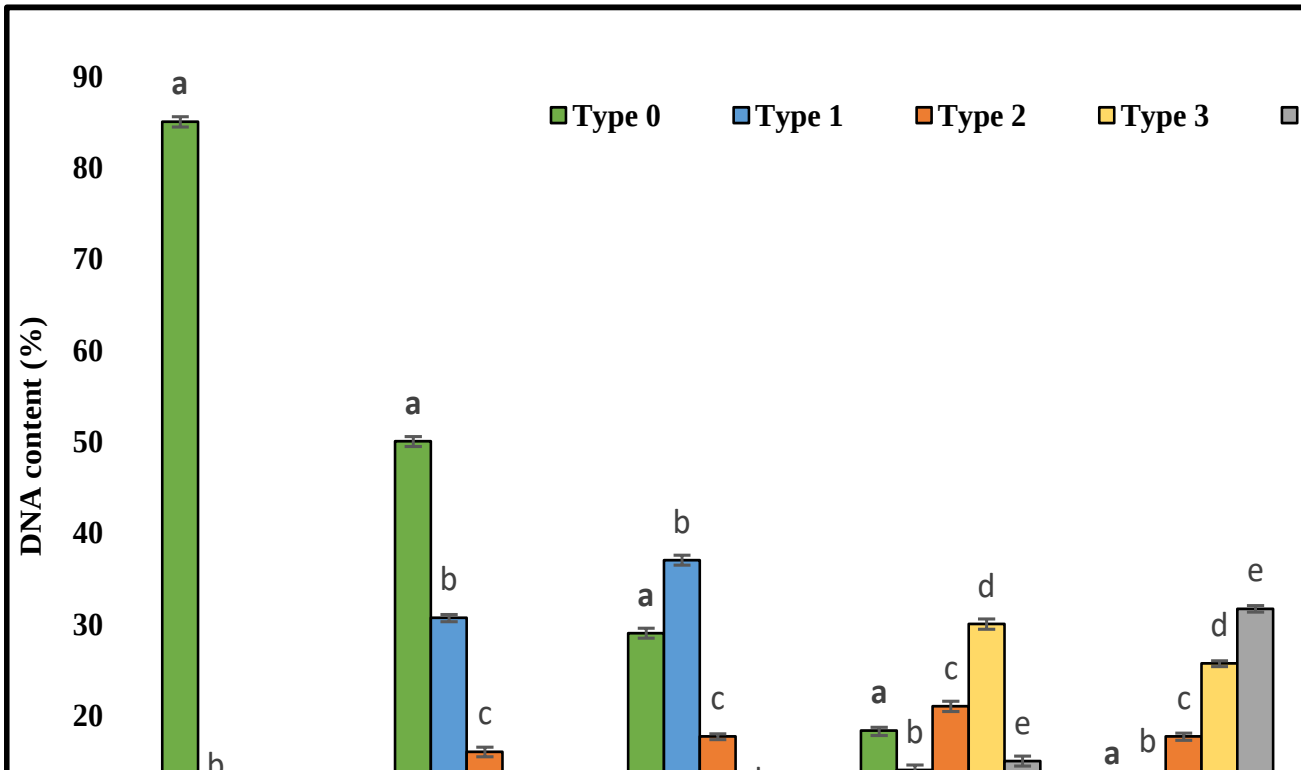

Figure 1. *DNA damage (percentage) in control and different exposed groups of fluoride & arsenic (separately & in combination) after 60 days. Alphabets represents significance (P<0.001) between different types of DNA damage (0 to 4). No significant difference was observed between different exposure groups Means bearing different letters are statistically significant from each other (based on Duncan's multiple - range test).*

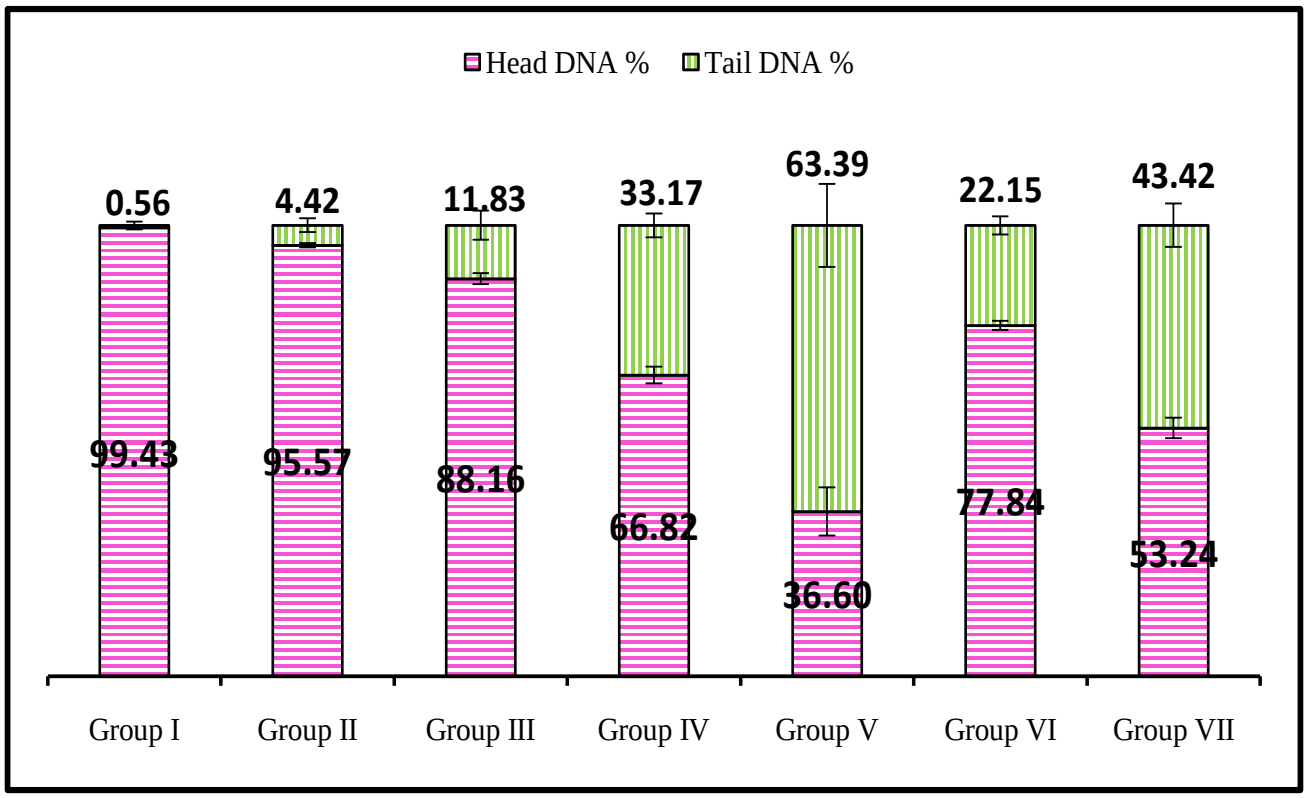

Figure *2. Percentage of DNA (Head and tail) in control and different experimental groups after 60 days of exposure*.

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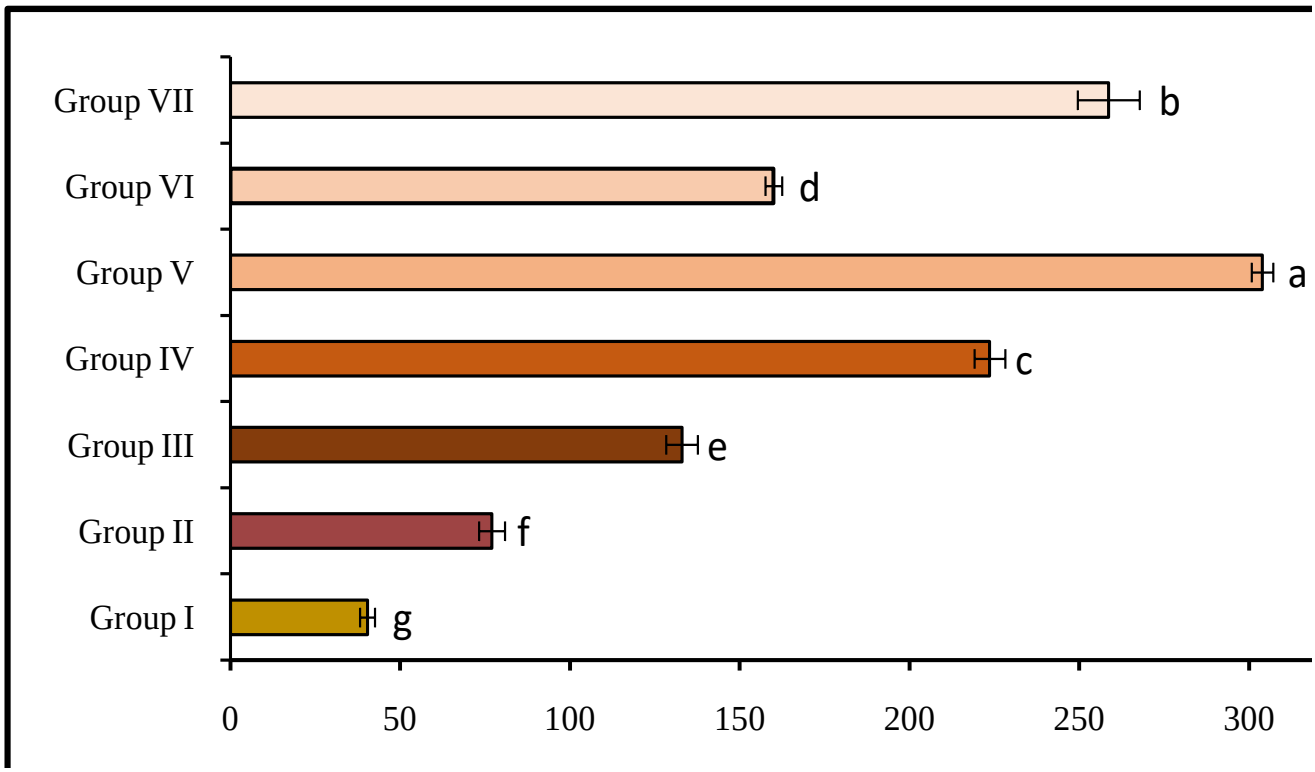

Figure *3. Comet length (pixel) in control and different experimental groups after 60 days of exposure. Alphabets represents significance (P<0.05) between different treatments.Means bearing different letters are statistically significant from each other (based on Duncan's multiple - range test).*

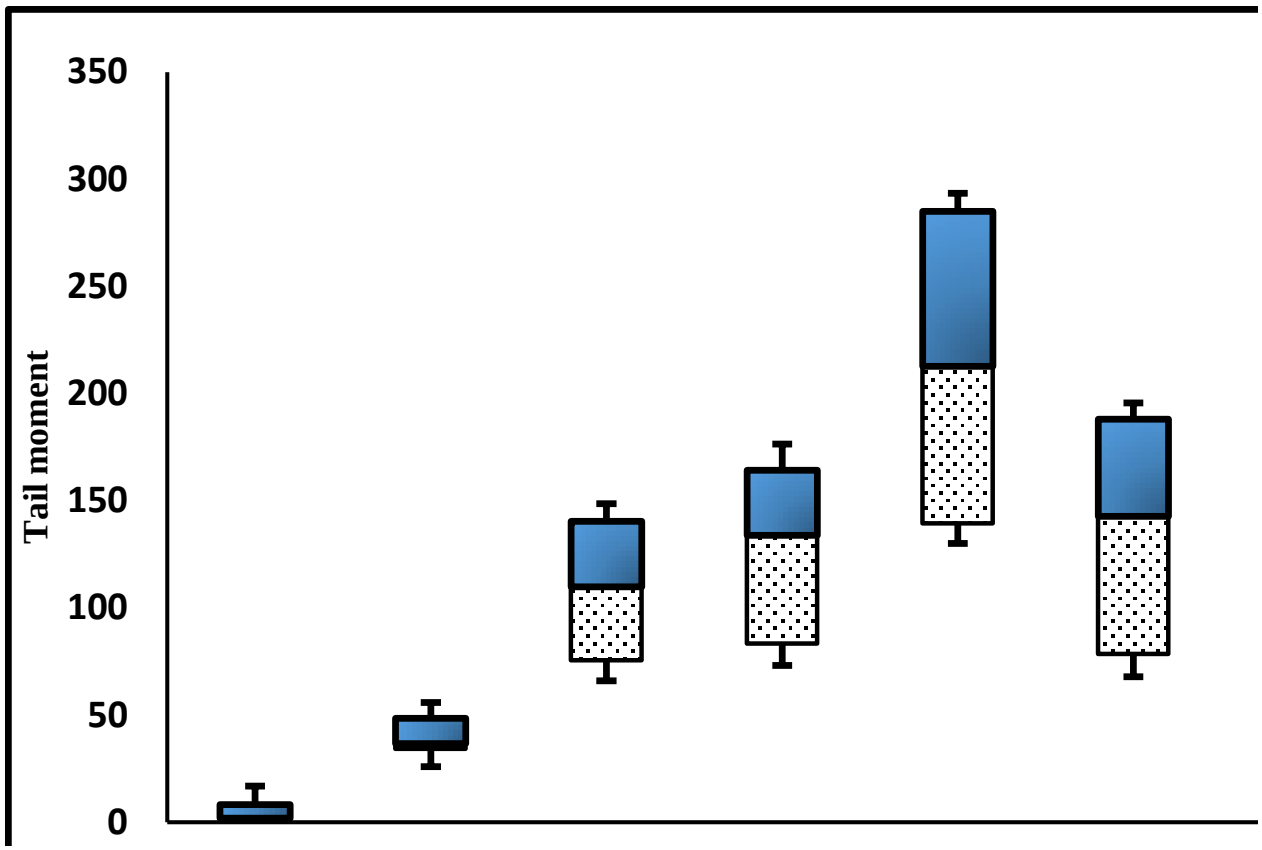

Figure *4. Tail moment (Tail DNA % * Tail length) in control and different experimental groups after 60 days of exposure*.

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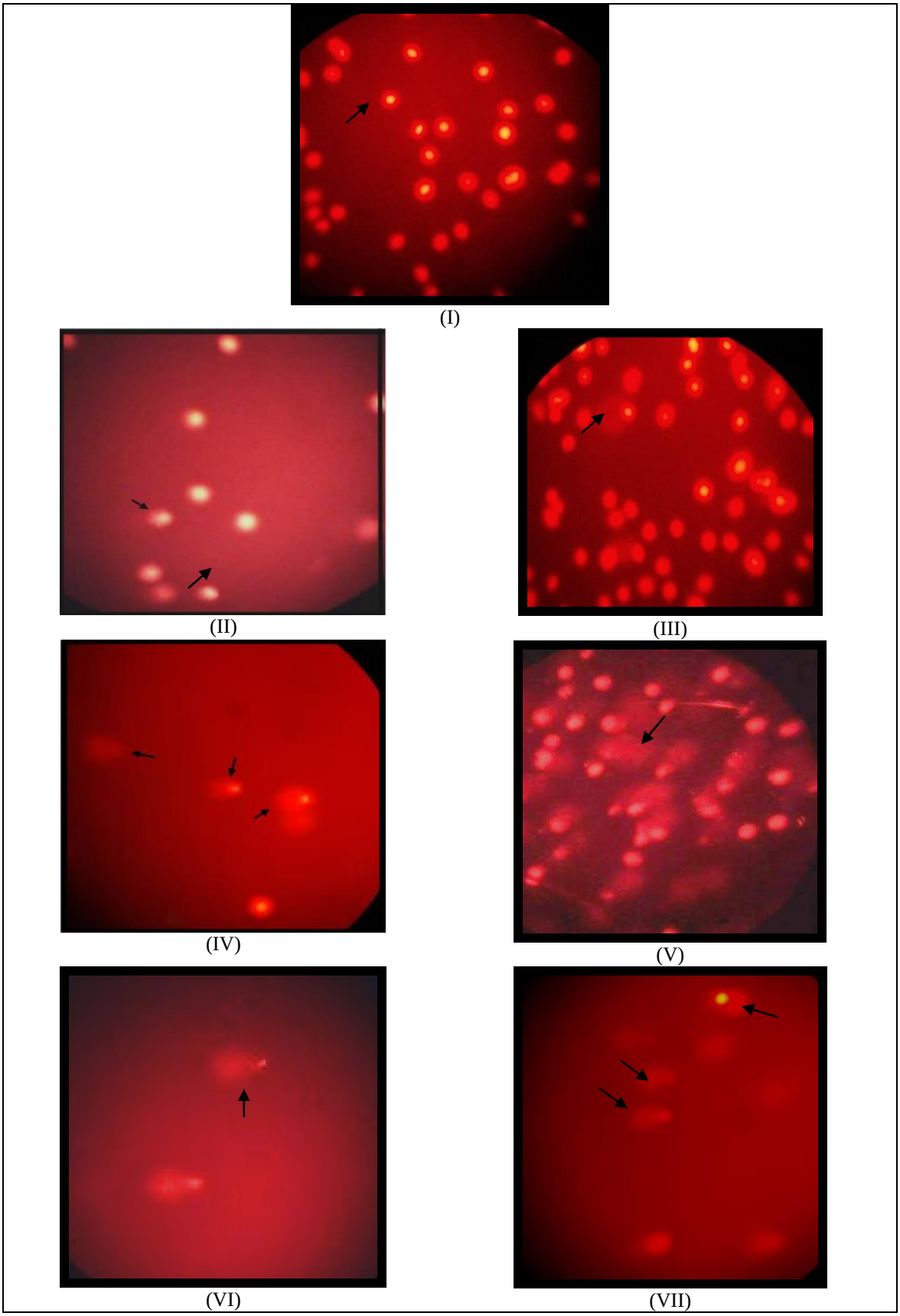

Figure 5. *COMET images, of blood cells collected from different groups (I to VII) after 60 days of exposure, observed under fluorescent microscope.*

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Discussion

Two commonly used techniques for testing genotoxicity in fish model are COMET assay and CAT (Chromosomal Aberration Test) [19]. In the field of ecotoxicology fishes considered as established models, where in the most reliable technique adopted for accessing DNA damage is COMET assay [20]. A study in *Clarias batrachus* reported increased Chromosomal aberration with increasing fluoride exposure [21]. Similar observations were reported in mice bone marrow, when tested for genotoxicity. Swiss albino mice were exposed to different sub lethal concentrations of sodium fluoride via drinking water, and breaks in chromosomal strands, appearance of micro nuclei was noticed [22]. Analogous experiments were conducted in Wistar rats, with a long-term exposure to fluoride, and analogous results were witnessed [23].

We agree with [24], considering arsenic a potent carcinogen; which he demonstrated using human lung cell lines and reported increased frequency of double stranded DNA breaks while performing Neutral COMET assay technique. Complimentary reports were established in *Oreochromis mossambicus* when exposed to different concentration of arsenic [25].

Antagonistic relationship between arsenic and fluoride was established by [26], where mice exposed to arsenic and fluoride separately has more DNA damage than to the group of mice with co-exposure of arsenic and fluoride, which is further supported by our investigations. In an experiment conducted by [27], arsenic and fluoride (singly and separately) were exposed to human peripheral lymphocytes cell cultures for 24h, ensuing DNA damage in all the exposed groups compared to control groups. Besides, comet length was found to be high in treated cultures; also, DNA percentage in head of comet was less and tail DNA% was more in exposed groups. However reverse condition was observed in non-treated groups. Antagonistic relationship between arsenic and fluoride is estimated in the present report and is further supported by the above study. Single strand breaks in DNA were observed in rat lymphocytes with the aid of Comet assay technique, when they were treated with arsenic, fluoride and co-exposure of arsenic & fluoride [28]. Comets with visibly long tails were seen in chronic fluoride (28 weeks) and arsenic treated groups in comparison to control, which are also analogous to the current work. Similar results were obtained [29], following exposure of fluoride and arsenic to human blood lymphocyte cultures for 24 hours. A noticeable lengthy comet tail was recognized in exposed cell cultures, indicating DNA damage.

Antagonistic relationship between arsenic & fluoride is due to formation of certain compounds like AsF_3, AsF_5 and AsF_6^- . Arsenic has an empty d orbital, giving it an affinity towards any electronegative element, like fluoride. Arsenic (III) undergoes SP_3 hybridization with fluoride and forms AsF_3 & when present in arsenic (V) go through SP_3d hybridization to form AsF_5, thereby reducing the individual effect of each [30].

V. CONCLUSION AND FUTURE SCOPE

Arsenic and Fluoride are two serious inorganic water pollutants worldwide. Extensive work has been done with reference to both fluoride and arsenic in combination, but a little has been explored in *Clarias batrachus.* Different views and reviews are obtainable for fluoride and arsenic relationship with each other. Our work has definitely brought clarity to their relationship between As & F^- when studied together. DNA damage was found be highest in arsenic exposed groups than in fluoride exposed groups and groups of $As+F^-$ exposure. Confirming the antagonistic nature of arsenic and fluoride, DNA damage was low in groups exposed to arsenic and fluoride together as compared to groups exposed to fluoride and arsenic individually. Estimation of accumulation status and biochemical effects of arsenic and fluoride would be an important tool for calculating ecological risk assessment.

ACKNOWLEDGMENT

The author is thankful to SOS Life sciences, Pandit Ravishankar Shukla University for support and providing lab facilities to carry out the required work.

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