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BENZO(A) PYRENE : RISK ASSESSMENT AND CHALLENGE IN *Clarias gariepinus*

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SUMMARY

In this study, evaluation of B(a)P hazards was applied on *Clarias gariepinus* in association with studying the validity of Diamond V probiotic as an immunostimulant factor against B(a)P toxic effects. Clinical, histopathological, immunological and cytological analyses were conducted and revealed the following: clinical alterations manifested by nervous signs, respiratory distress, mortality rate 25% with detectable reduction of mortality and health improvement in association with probiotic application. Histopathological alterations exemplified by degenerative tissue changes, tissue necrosis and hyperplasia with no detectable histopathological probiotic role of improvement were observed. Using of *in vitro* single cell gel assay indicated the hazard implementation of B (a)P on derived erythrocyte cells, sharing the same result of micronucleus assay. The role of action of Diamond V showed a reduction in the induced micronuclei when challenged the B(a)P, this also was indicated through the immunological outcomes that revealed the suppressive effect of B(a)P on serum lysozyme, nitric oxide, globulin and total proteins levels associated with the long term B(a)P exposure, these reduced levels were found to be elevated in case of Diamond V treatment and to some extent in case of mixed B(a)P and Diamond V treatment. It could be concluded that, B(a)P had a drastic cytotoxic effect on *Clarias gariepinus*. Also, yeast culture probiotic is recommended as a cytoprotective and immunostimulant feed additive in cultured fishes.

Keywords: B(a)P, Risk assessment, genotoxicity, Cytotoxicity control, Probiotics, *Clarias gariepinus*.

INTRODUCTION

Environmental pollutants have been reported to accumulate in freshwater invertebrates and fish and have threatened human health, either directly or indirectly, through the food chain (Porte and Albaiges, 1994; Dixon et al., 2002 and Jacobs et al., 2002). Accumulated compounds have been the subject of growing interest, and many have been found to be harmful and carcinogenic.

The hypothesis of stimulation of the immune system of aquatic organisms against pollutant stress may be also considered. Many immunostimulants of microbial cell wall origin have been tested on fish and shellfish, eg. glucans (Song and Huang, 2000; Bricknell, and Dalmo, 2005)

The concept of biological disease control, particularly using microbiological modulator for disease prevention, has received widespread attention. A bacterial supplement of a single or mixed culture of selected non-pathogenic bacterial strains was termed probiotics.

In aquaculture, nutritional modulation as probiotics (yeast culture) has been developed to enhance host defense mechanisms against pathogens by increasing phagocytosis, antibody production, increasing the chemiluminencent response and superoxide anion production (Sakai, 1998).

Diamond V which was selected as a probiotic in this study is a yeast fermented product which contains fermentation metabolites which stimulate the growth of digestive bacteria in the rumen and intestinal tract of the animal. It does not depend on live microorganisms to be functional. The Yeast Culture metabolites are very stable in feeds and mineral mixes, with no special handling required. Since the yeast has already produced the growth factors during the fermentation process, yeast viability in the feed is not a concern. Pelleting has little effect on the activity of the fermentation metabolites. So, Diamond V can be used effectively in pelleted feeds without concern for loss of effectiveness. Yeasts are promising candidates as probiotics, because of their abilities to produce polyamines and to adhere and grow in the intestinal mucus of fish. Polyamines are ubiquitous molecules that participate in numerous biological processes including cell replication and differentiation, and biosynthesis of nucleic acids and proteins .The role of dietary polyamines in mammals has been studied extensively. But literature concerning their effects on fish is scarce (Askarian et al., 2006).

The impact of the environmental contamination on fish immune system is of economic relevance for fishes as well as aquaculture. polycyclic aromatic hydrocarbons (PAHs) such as Benzo (a) pyrene (B(a)P) known to induce alteration of immune functions (Hoffman et al. 1999). As, they

reduce antibody responses (Thuvander and Carlstein 1991) also decrease the number of circulating lymphocytes (Khan, 2003).

B(a)P was selected in this study as a challenging immunosuppressive & cytotoxic agent because of its importance as a dangerous mutagenic and carcinogenic polycyclic aromatic hydrocarbon (PAHs), appears to have a wide distribution in the aquatic environment (Culp and Beland, 1994) and also the detection of this toxic chemical is served as an indicator of other PAHs which contaminate the environment (Irwin et al., 1997).

B(a)P has been used extensively as a positive control in a variety of short-term mutation tests. It was active in assays for DNA repair and mutation in bacterial cells; mutation in fruit flies; DNA binding, DNA repair, sister chromatid exchange (SCE), chromosomal aberrations, point mutation and transformation in mammalian cells in culture; and in *in-vivo* tests in mammals, including DNA binding, sister chromatid exchange, chromosomal aberrations(IARC, 1983).

So, this study was designed firstly to prove the cytotoxic effects of B(a)P in *Clarias gariepinus* during short term and long term exposure through recording the clinical and histopathological alterations, immunological indicators, comet assay and micronucleus assay and secondary to investigate the role played by a yeast culture probiotic against B(a)P cytotoxicity during long term exposure .

MATERIALS AND METHODS

Experimental fish:

In the present study two hundreds (200) *Clarias gariepinus* were collected from different fish markets at Giza. The average body weight was 200 ± 50 g. The fish were apparently healthy & free from any visible lesions. They were transported to the Fish Disease and Management laboratory, Faculty of Vet. Med., Cairo University, in large plastic water containers. In the laboratory, fish were acclimated in the glass aquaria for two weeks, prior to the experiments.

Benzo(a)Pyrene: A lyophilized pure powder was purchased from SIGMA Inc.Co. It has a yellowish colour and faint aromatic odour. It is insoluble in water ,sparingly soluble in alcohol but soluble in ether, benzene ,toluene, xylene and dimethyl sulphoxide (DMSO). B(a)P has a molecular weight 252.3 and molecular formula $C_{20}H_{12}$.It has a melting point of 179.0-179.3°C and boiling point 495°C.

Dimond-V XP yeast culture: is a yeast fermented product which contains fermentation metabolites. Diamond V® is a registered trademark of Diamond V Mills, Inc. © 1997 by Diamond V Mills, Inc. All rights reserved. Diamond V Mill

Inc. 838 1st Street NW Cedar Rapids, Iowa 52407-4570. List of ingredients: *Saccharomyces cerevisiae* yeast grown on a media of ground yellow corn, hominy feed, corn gluten feed, wheat middlings, rye middlings, diastatic malt and corn syrup, and cane molasses.

Determination of 96 hours half lethal concentration (LC_{50}) B(a)P:

Estimation of the concentration of B(a)P which is most likely to cause 50% mortality as (LC_{50}) for 96 hours exposure was calculated according to the method described by Behrens and Karber (1953).

Experimental design:

1-Short term exposure:

A total of 45 *Clarias gariepinus* with an average body weight of 150g were collected , transported and acclimated to laboratory conditions in aerated, and dechlorinated tap water in 150 l aquaria for 1 week, at 28+2°C, under a natural photoperiod (light/dark hours = 12/12). Fish were divided into three equal groups one exposed to 0.500µg/l or 0.5ppm (1/2 LC_{50}) B(a) P (dissolved in DMSO) to evaluate its toxicity over an exposure period of 7 days; in addition to two other groups; control water and control DMSO. The aim of the toxicity evaluation was achieved through the investigation of clinical signs, histopathological changes and micronucleus assay.

2- Long term exposure:

The long term toxicity case experiment was carried out in two trends: First to evaluate the pollutant toxicity compared with the short term exposure, the second is to evaluate the validity of the application of probiotic (artificial diet supplemented with a probiotic) as a cytoprotective and immunostimulant agent against a proved cytotoxic and immunosuppressant agent (B(a)P). Hundred (100) fish of almost the same weight and size were divided into five groups and stocked into the twenty aerated glass aquaria. The duration of the experiment was 4 weeks where fish were maintained at the same temperature and oxygen of the acute case of exposure. During this period, every clinical abnormalities and fish mortality were recorded. Water for each group was periodically changed every 3 days. Fish were fed at a rate of 3 % of their body weight/day using commercial fish diet containing 30 % protein. Probiotic mixed artificial pellet was prepared (10g probiotic/Kg artificial diet). Fish were reweighed every 2 weeks for ration adjustment. At the end of the experimental period (4 weeks) fish were scarified to obtain the sera and internal organs. Individual serum samples were kept frozen at -20 °C for further investigation. Histopathological, cytological and immunological analyses were carried out for realizing the study purposes.

Clinical investigation and Post Mortem examination:

The exposed fish were kept under proper observation during the period of experiment for any external clinical abnormalities , PM lesions or deaths according to the method described by Amlacher (1970).

Histopathological studies:

Tissue specimens from skin, muscles , gills, liver, kidneys, spleen, intestine, ovary and testis were taken from *Clarias gariepinus* that were exposed to B(a)P in water by the end of each exposures. The samples were fixed in 10% formal saline, processed by conventional method, sectioned at 4 µm and stained with Haematoxylin and Eosin (Bancroft et al., 1996 ; Roberts, 2001).

Immunological studies:

Both the immunosuppressive effects of B(a)P and the immunostimulant effects of the yeast culture probiotic were evaluated through:

1-Lysozyme lysoplate assay:

Serum lysozyme activity was determined using a micrococcus lysoplate agar assay developed by Osserman and Lawlar (1966). 15 µl of the collected serum samples at 1st and 2nd weeks post treatment were poured into the depressions of the agar plates and were incubated for 16 hours at 37°C. The lysozyme activity was calculated using logarithmic regression analysis and hen egg lysozyme as standard.

2 - Nitric oxide (NO) assay:

At 72 hours post treatment, the nitrite level in the collected serum samples was calculated according to the method described by Green et al. (1982).

3- Serum total proteins

Serum total protein was detected according to Biuret tecknique after Doumas et al.(1981), serum albumin according to Doumas et al. (1971), serum globulin was obtained by subtraction of the obtained albumin value from the serum total protein, albumin globulin ratio was calculated.

Cytological study:

This study was conducted using two trends of investigation:

A- The *in vivo* investigation: (Evaluation of DNA damage by micronucleus assay) in case of short and long term exposures:

A drop of blood from the gills of *Clarias gariepinus* exposed to B(a)P in water was obtained by the end of both exposures. It wa mixed with a drop of fetal calf serum on a clean dry slide and air-dried. The specime was fixed in methanol for 5 minutes. Slide were stained with 10% Giemsa stain for 1 minutes. One thousand erythrocytes were e amined for every fish to determine the percen age of cells containing micronuclei (Al-Sab and Metcalfe, 1995).

B- *In-vitro* investigation: (Evaluation of DNA damage by comet assay) :

In this trend of investigation, the single cell gel electrophoresis was applied for toxicity assessment of B(a)P using three concentrations (0.1, 0.5, 1.0µg/l). The comet assay was conducted with whole blood under yellow light to prevent UV induced DNA damage and performed with slight modifications on the method described by Speit and Hartmann (2004). A fluorescence microscope (400 x magnification) was used for slide analysis. Fifty cells per concentration were randomly scored and the percentage of the comet cells were calculated.

RESULTS AND DISCUSSION

The discharges of unpurified sewage and wastewater has resulted in accumulation of contaminants in freshwater sediments. Influx of contaminants, such as polycyclic aromatic hydrocarbons (PAHs), various ecosystem-disrupting materials, into freshwater has a destructive effect on communities and on individual freshwater organisms. Probiotics principally inhibit the growth and decrease the pathogenicity of the pathogenic bacteria, enhance the nutrition of the aqua-cultured animals, improve the quality of the aquaculture water and decrease the use of antibiotics and other chemicals; thus decreasing environmental contamination by the residual antibiotics and chemicals. This benefit of probiotics will be long lasting, and the application of probiotics will become a major field in the development of aquaculture productivity in the future.

To evaluate the toxic effects of B(a)P on *Clarias gariepinus*, it was necessary to determine the half lethal concentration (LC_{50}) of B(a)P. The recorded LC_{50} of B(a)P was 1ppm. Dimethylsulfoxide (DMSO) was used as a solvent for B(a)P because it has no toxic effects on fish within the concentration used as demonstrated by many investigators (Stephen et al., 1967). This result is more or less similar to that of Marzouk et al., 2005 who recorded that the LC_{50} of B(a) P/96h in *O.niloticus* was 1.2 ppm and this difference may be due to fish breed.

The recorded clinical signs in this study were more or less similar in both B(a)P short term and long term exposed groups while was less severe in group of fishes which exposed to B(a)P for long term and fed on diet supplemented with a yeast culture probiotic. Clinical signs were manifested in the form of nervous manifestations, , abnormal swimming behaviour in the form of erratic swimming and/or circling was also noticed especially at the start of exposure to B(a)P and disappeared by time and abnormal skin discloura-tion in few cases and darkness in the others. The postmortem findings revealed, congestion and haemorrhages in all internal organs in addition to pale anaemic gills.Mortality rate(25 %) was recorded in case of long term B(a)P exposed group while it was only 10 % in fish group which

exposed to B(a)P for long term and fed on diet supplemented with a yeast culture probiotic.

Nervous manifestation and abnormal skin pigmentation might be attributed to the direct damaging effect of B(a)P on the nervous mechanism controlling pigmentation. In this aspect, this explanation is supported to the finding of Tu et al. (2002) who found that, B(a)P had a direct neurotoxic effects on the nervous tissue of exposed mice.

The recorded reduction in mortality rate in fishes exposed to B(a)P and received diet supplemented with probiotic may be due to the capability of the probiotic to antagonize the toxic effects of B(a)P and to the immunostimulant effect of this probiotic which recorded in this study. Concerning this aspect, Scholaz et al. (1999) found that the whole cells and the b-glucan of *Saccharomyces cerevisiae* improved the resistance of juvenile paenids to vibriosis and led to improvement of of larval survival.

The histopathological changes which have been found in various organs, their frequencies and severity were increased with increasing the concentration and the duration of exposure to Banzo (a) pyrine. Also the histopathological finding after application of probiotic as an immunostimulant with the chronic exposure were recorded.

No histopathological changes were demonstrated in both the control group and the probiotic group.

Concerning the histopathological findings in the skin in short term exposed group, no pathological changes were demonstrated but in the chronic one vacuolation and necrosis in the club cells and hyperactivation of mucous secreting cells in the epidermal layer and hyperactivation of melanophores in some cases associated with oedema in between the dermal layer were recorded. These findings (Fig. 1) were in agreement with that of Abbas (2006).

The microscopical examination of the gill tissue in short term exposed group revealed distortion of the secondary lamellae which distributed focally along its length associated with degenerative changes in the form of hydropic degeneration mainly of respiratory epithelium lining th secondary lamellae and devoid of these cells du to their necrosis. But in long term exposed group more advanced changes manifested by epithelia hyperplasia and lamellar fusion of secondary la mellae leading to obliteration of inter-lamell space could be demonstrated (Fig, 2). The re corded changes were in agreement with that re corded by Marzouk et al. (2005).

Regarding the histopathological findings in t hepatic tissues of short term exposed group vealed cellular changes in the form of degene tion and necrosis. One of these changes w fatty degeneration in some of hepatocytes .T cells were swollen with abundant fat content a exhibited a distorted pyknotic nuclei.Congest

also detected in the hepatic blood vessels and sinusoids. Also there was hyperplasia in the bile ducts associated with periductal fibrosis and necrosis within the lining epithelium of the duct (Fig.3). These records are in agreement with those of Hinton and Lauren (1990). They mentioned that there was a close association between hepatocytes and the billiary system in the teleost liver which is responsible for the high incidence of peribilliary damage to hepatocytes often visible in fish exposed to chemicals. Focal aggregation of foamy cells as indicators of fat metabolism disturbance were detected in between the hepatic tissue (Fig 4). These foamy cells may also resulting from disturbance in fat metabolism where in the process of cholesterol transport, apo B-containing VLDL particles are released to the circulation from the liver, and through metabolism by endothelial lipoprotein lipases (LPL) and hepatic lipases (HL), are catabolized into LDL particles. Once these LDL particles have traversed arterial endothelial wall (along with their associated cholesterol),the particles may become modified and undergo uptake by subendothelial macrophages,which express specialized or scavenger receptors (SR). The result is the formation of cholesterol-laden macrophages (otherwise known as foam cells).

Changes in short term exposed group revealed that the liver suffered from sever fatty degeneration, the cells were compressed against each others with pyknosis of the nuclei (Fig.5). The obtained results were in agreement with that of Gingerich (1982) who pointed out that the teleost liver may be susceptible to chemical damage because of the relatively slow hepatic blood flow in fish relative to cardiac out put.

Concerning the histopathological changes in the renal tissues in short term exposed fish group, mild degenerative changes in the tubular epithelium of some renal tubules in the form of vacuolar degeneration were recorded. But more advanced changes were recorded in long term exposed group where the degeneration and necrosis were not restricted to convoluted tubules but extended to involve the collecting tubules where there were hyperplasia and necrosis of the epithelial lining of the collecting tubules (Fig 6). These changes were associated with thrombosis of the renal blood vessels, also infiltration of individual numbers of inflammatory mononuclear cells in between the interstitial tissue (Fig,7).The renal tissue degeneration were explained by Lemaire et al. (1990) who stated that kidney have high B(a)P metabolism rate and high levels of metabolites are found in kidney tissue which leading to its degeneration.

The microscopical investigation of the spleen in short term exposed group revealed focal areas of necrosis and depletion of the spleenic haemapoietic tissues in some cases. These records may be explained according to Teles et al. (2003) who found that B (a) P exposure caused an elevation

of corticosterol level in the circulation which leading to switch of the metabolic pathway of fish from anabolic state to catabolic state also the corticosterol plays a role in immuno-suppression through reduction of the number of circulating lymphocytes and depletion of the lymphocytes population from the spleen.

In long term exposed group there was hyperactivation of melano-macrophage centers also diffuse infiltration with melanophores within the spleenic tissue which are polyhedral large cells appeared brownish or dark brown in colour (Fig. 8) .The intensity of coring cells increased in melano-macrophage centers as a result of stress condition where the ACTH (adreno - corticotrophic hormone) released. This hormone produces a rapid dispersion of melanin granules in many fish species, thus pigment changes could be attributed to high level of ACTH associated with stress condition (Gardner et al., 1989 and Hanna, 2004).

No pathological changes were detected in the intestine and ovary. Otherwise, testis in short term exposed group showed selective necrosis within many spermatocytes in the seminiferous tubules in some cases (Fig. 9), but no other changes were demonstrated in long term exposed group.

Pathological changes in this study were not enough to explain the role of probiotics in protecting fish or decreasing the pathological changes in different organs of fish, this may be due to the short duration of probiotic application and further study of long term application of probiotic is needed to prove or negate its effect on the histopathological changes.

Regarding the results of immuno parameters during long term B(a)P exposure and challenging with a yeast culture probiotic, serum lysozyme concentration was initially increased in fish fed only yeast- culture at 1st week post treatment (Fig., 10) which has become significant at 2nd week post treatment however, fish that fed both yeast culture and B(a)P showed relative elevation of lysozyme concentration in comparison to the control. In contrast, those, which had been exposed to B(a)P alone, induced suppression of lysozyme concentration at 1st week, being more at 2nd week post exposure.

The data shown in (Fig., 11) revealed that serum NO concentration was significantly reduced in fish exposed to B(a)P alone 72 hours post exposure when compared to control one but this result was significantly higher in both fish that fed yeast - culture alone and or in combination with B(a)P respectively.

At the end of experiment, serum globulin and total proteins were lower in fish that only exposed to B(a)P wherever those fed yeast-culture alone or with B(a)P expressed higher values but considered non- significant as been illustrated (Table, 1).

The exposure of *Clarias gariepinus* to B(a)P alone resulted in decline of both serum lysozymes and NO concentration. Our findings were in agreement with those mentioned by Price et al. (1997) showed a decrease in these two immune parameters in carp exposed to pollutants compared to control fish. Moreover, Charles (1995) stated that the oxidative burst activity of macrophages to generate reactive oxygen species as reflected by NO was suppressed 7 days after exposure. Thus, the inhibition of both serum lysozymes and nitric oxide could be attributed to reduced phagocytic functions.

It is well established that yeast culture are recognized by receptors expressed on fish monocytes / macrophages and able to stimulate fish innate response by increasing production of both nitrogen and oxygen intermediates by phagocytes which acting as potent microbicidal agents (Brown and Gorden, 2003). Also, yeast culture may hypothetically participate in autocrine stimulation of lysozyme production (Robertson et al. 1994 and Jang et al. 1995). An enhanced lysozyme activity within 2 weeks of feeding with yeast culture also detected by (Yoshida et al. 1995 and Irianto and Austin 2002).

In this experiment, activation of non specific immune response as suggested by an increase of lysozyme activity seems to be slightly correlated with serum total proteins perhaps by production of immunoglobulin. This result was also described by (Zilberg and Klesieus , 1997). Therefore, the results in the current study demonstrate the immuno-enhancing ability of yeast culture that might lead to benefits to the host by detoxifying toxic agents from the surrounding environment in aquaculture.

Micronucleus assay can be used as a genotoxicological test-system in fishes since some particularities were observed (Grisolia, 2002).The frequencies of micronucleus (Mn) in Clarias gariepinus gill cells by B(a)P exposure in this study are shown in (Table, 2) for short and long term B(a)P exposures. Significant increase in the frequency of Mn compared to the control water and the control of DMSO were recorded in case of both short and long term exposures. Figure,12 shows the induction of the Mn by B(a)P exposures.

The applied probiotic showed a role as an antimutagenic which was indicated in this report when added with the B(a)P, where the frequency of the Mn decreased in case of long term B(a)P exposed group compared to those exposed to B (a)P and received a diet supplemented with probiotic).

Results of micronucleus are more or less similar to those reported by Marzouk et al. (2006) in which a significant increase in Mn frequency within a dose dependent manner in association with both acute and chronic B(a)P exposures in

O. niloticus was proved. In this study, the recorded increase in Mn frequency in 0.1ppm B(a)P exposed group compared to 0.5ppm B(a)P exposed group could be explained by the foundation of Teles et al. (2003) who observed that the genotoxic response profile of *Anguilla anguilla* L. to naphthalene may reflect a considerable DNA repair capacity and or a metabolic adaptation providing an efficient naphthalene biotransformation and detoxification. The antimutagenic effect played by the yeast culture probiotoc through a reduction in Mn frequency is supported that of Marotta et al. (2003) who concluded that effective probiotics treatment, through mechanisms still to be fully elucidated (decreased fecal pH, specific reduction of carcinogenetic bacterial enzymes, modulation of gut-associated and systemic immune system etc.) has the potential to exert significant antimutagenic properties against colon cancer.

The measurement of cytogenetic alterations *in vitro* is considered an initial step in the risk assessment procedures for genotoxic agents. The concern about genotoxic pollutants in natural fish population makes the use of fish-derived cells a useful tool for these purposes. Reactive intermediates of PAHs metabolism can bind to DNA causing damage. As a PAH model in chemical carcinogenesis studies, B(a)P metabolic activation that leads to formation of stable DNA adducts, oxidative damage, depurination and strand breaks is well documented (Phillips, 1983; Stansbury et al., 1994; Cavalieri and Rogan, 1995 and Devanesan et al., 1996). Estimates of the relative rates of DNA damage indicate that single strand breaks are the most prevalent type of damage (Bernstein and Bernstein, 1991). Measured strand breaks can be a reflection not only of direct strand breakage, but also of alkali labile sites and of repair enzyme-mediated breakage giving the information about exposure of organisms to wide range of genotoxic chemicals and genotoxic environmental mixtures. Early study concerning prediction of carcinogenic/mutagenic potential, revealed that the production of strand breaks is correlated with the carcinogenic and mutagenic properties of environmental contaminants with diverse structures (Sina et al., 1983). It was also demonstrated that various isolated cells from aquatic species respond to a range of direct and indirect genotoxins by DNA strand breaks formation (Mitchelmore and Chipman , 1998).

The DNA lesion leads to incomplete transcription, cellular dysfunction, growth inhibition, aging, weakened immunity and diseases in the organism itself. Furthermore, it has created a severe problem within ecosystems and the food chains (Kurelec and Gupta, 1993). However, every organism has a recovery mechanism against severe DNA lesion, and DNA strand breaks necessarily occur during the recovery process of DNA damage. DNA strand breakage itself is not the DNA lesion caused by UV or exposure to pollutants but a result of the recovery process. Consequently, observation of DNA strand breaks suggest the existence of various DNA lesions (Unal et al 2004). Measurement of DNA damage is one of the most important indicators in detecting DN

lesion and methods, such as alkaline elution (Bolognesi et al., 1996), alkaline unwinding (Daniel et al., 1989) and alkaline precipitation (Gagne et al., 1995), have been developed, including single cell gel electrophoresis. The recently developed single cell gel electrophoresis method could detect DNA single strand break at a single cell level, as well as incomplete DNA excision regions, during the DNA lesion recovery process (Koppen and Angelis, 1998 and Mohankumar et al., 2002).

The comet assay used in the current study is applicable to any cells, including nuclei, and can be successful with a few microliters of blood from target organisms without sacrifice of experimental animals. In this study, the level of DNA damage is revealed by the tail moment defined as the product of tail length and fraction of DNA in the tail (Helma and Uhl, 2000). Fig. 13 and table, 3 represents the comet assay results for whole blood from *Clarias gariepinus* exposed to three doses of B(a)P for different exposure times (1 , 2 and 3 hr.). *Clarias gariepinus* blood cells showed a high frequency of DNA damage in a manner of dose- dependent and time exposure. An observed increased tail moment at the highest concentration tested (1.0µg B(a)P) when compared to the untreated controls. At the lowest concentration exposure, there was no significant alteration in the levels of DNA damage along the exposure period.

Our results were in accordance with Seonock et al. (2006) in a study revealed that, DNA breakage were recorded between flounder (*Paralichthys olivaceus*)cells exposed to B(a)P in two doses for two days, the mean tail lengths increased significantly in a dose dependent manner. The author in their study demonstrated the comet assay as a successful tool in monitoring contamination and assessing genotoxicity of PHCs in marine organisms either *in vitro* or *in vivo*.

However, B(a)P confirmed its considerable DNA damage effect through the *in vitro* comet assay, and its induction of DNA strand breakage .

In conclusion, this study has demonstrated that B(a)P had a drastic cytotoxic effects on *Clarias gariepinus* manifested through the recorded clinical and histopathological alterations, immunosuppressive effects and DNA damage. The current study of the detection of DNA damage resulting from contaminant exposure will be a key tactic in assessing the general health of freshwater organisms, identifying the importance of genotoxicity, or predicting the effects on populations or communities in the aquatic system. The yeast culture probiotic applied in this study had a protective role against B(a)P cytotoxicity proved through its ability to reduce the mortality rate, immunostimmulant effect and antimutagenic effect. So, yeast culture probiotic is recommended as a cytoprotective and immunostimulant feed additive in cultured fishes. Further long term studies are needed to support the recorded results.

Table (1): The effect of B(a)P , probiotic and both on serum proteins.

Parameters / Treatments	Total protein	Albumin	Globulin	A/G ratio
B(a)P(0.1ppm ,4ws)	5.83 ± 0.03	1.14 ± 0.11	4.69 ± 0.08	0.24 ± 0.03
Probiotic(10g/Kg diet,4ws)	9.40 ± 0.43	3.08 ± 0.16	6.33 ± 0.35	0.51 ± 0.05
B(a)P 0.1ppm+ Probiotic(10g/Kg diet,4ws)	6.98 ± 0.78	1.89 ± 0.02	5.10 ± 0.77	0.89 ± 0.06
Control	7.47 ± 0.19	2.70 ± 0.02	4.78 ± 0.18	0.56 ± 0.02

Data presented as means ± SE (n = 10).

Table (2): Mean values and percentages of micronuclei in case of short and long term exposure of *Clarias gariepinus* to B(a)P.

Treatments	Total No. of examined fish	Total No. of PCE,*	Total No. of Mn.	Micronucleus	
				Mean ± S. E	%
Control	5	5000	17	(3.4 ± 0.2)	0.34
DMSO	5	5000	18	(3.6 ±0.22)	0.36
B(a)P(0.5 ppm,1w)	5	5000	70	(14 ± 0.548)	1.4
B(a)P(0.1ppm,4ws)	5	5000	84	16.8 ± 0.37)	1.68
B(a)P(0.1ppm+probiotic 10g/Kg diet 4ws)	5	5000	47	(9.4± 0.245)	0.94
Probiotic(10g/Kg diet,4ws)	5	5000	15	(3.0 ± 0.18)	0.3

Table (3): Detection of DNA damage frequency in blood erythrocyte cells of *Clarias gariepinus* exposed *in vitro* to B(a)P:

Treatments	Damage Frequency %		
	1 hr	2hr	3hr
Control	1.00±1.3	1.5±2.3	2.6±0.9
B(a)P 0.1 µg/L	3.6±2.1	5.2±1.2	7.1±2.6
B(a)P 0.5 µg/L	9.7±4.6	12.1±2.6	17.4±4.5
B(a)P 1 µg/L	19.8±0.7	35.9±1.7	72.9±3.7

Data presented as means ± SE (n =50)

Fig.1: A micrograph showing hyperactivation of mucous secreting cells in the epidermal layer and hyperactivation of melanophores associated with oedema in between the dermal layer) in skin of *Clarias gariepinus* exposed to 1/2 of LC_{50} of B(a)P for 1week. (H&E, x400).

Fig.2: A micrograph showing epithelial hyperplasia and lamellar fusion of secondary lamellae leading to obliteration of inter lamellar space in gills of *Clarias gariepinus* exposed to 1/10 of LC_{50} of B(a)P for 4 weeks. (H&E, x 200).

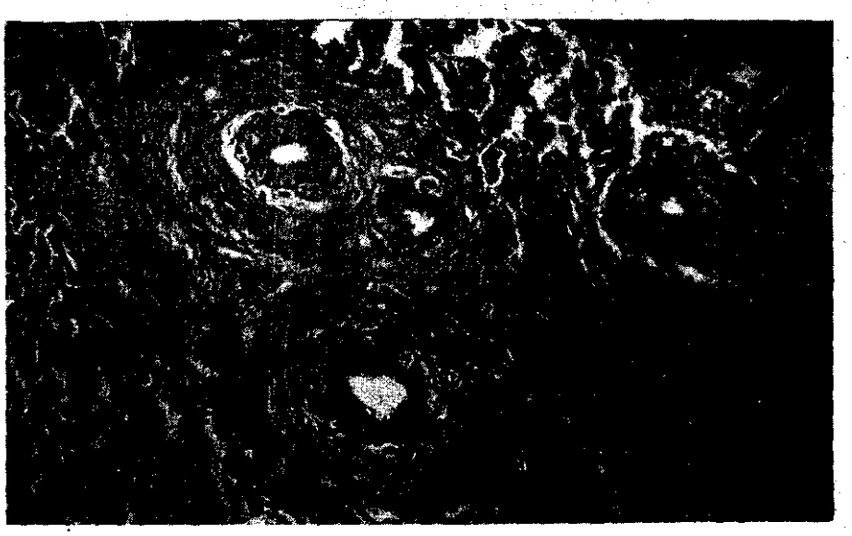

Fig.3: A micrograph showing hyperplasia in the bile ducts associated with periductal fibrosis and necrosis within the lining epithelium of the duct in Liver of *Clarias gariepinus* exposed to 1/2 of LC_{50} of B(a)P for1 week. (H&E, x 400).

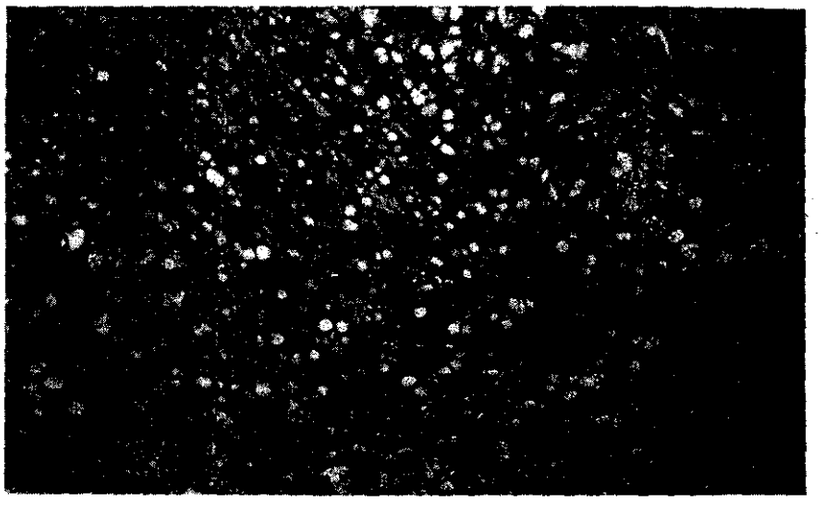

Fig.4: A micrograph showing focal aggregation of foamy cells. The cells were detected in between the hepatic tissuein liver of *Clarias gariepinus* exposed to 1/2 of LC_{50} of B(a)P for1 week. (H&E, x 400).

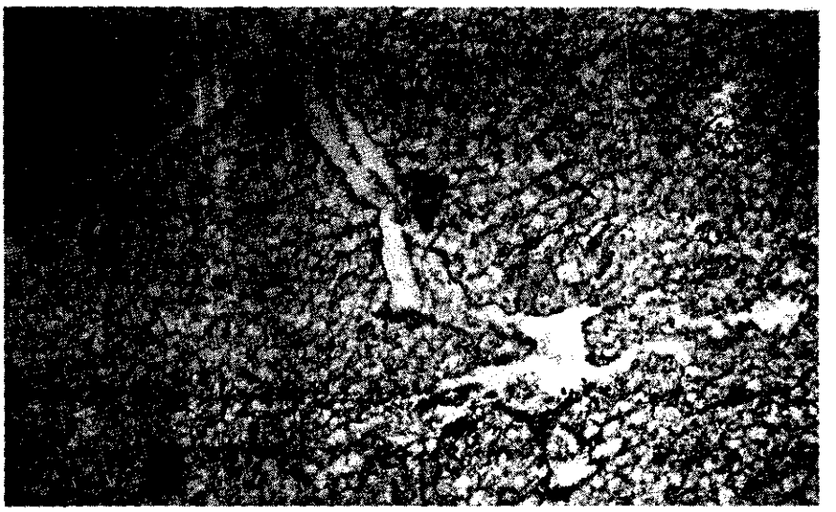

Fig.5: A micrograph showing liver suffered from sever fatty degeneration in *Clarias gariepinus* exposed to 1/10 of LC_{50} of B(a)P for 6 weeks. (H&E, x400).

Fig.6: A micrograph showing hyperplasia and necrosis of the epithelial lining of the collecting tubules in the kidney of *Clarias gariepinus* exposed to 1/10 of LC_{50} of B(a)P for 6 weeks. (H&E, x 400).

Fig.7: A micrograph showing thrombosis of the renal blood vessels, also infiltration of individual numbers of inflammatory mononuclear cells in between the interstitial tissue in the kidney of *Clarias gariepinus* exposed to 1/10 of LC_{50} of B(a)P for 6 weeks. (H&E, x 400).

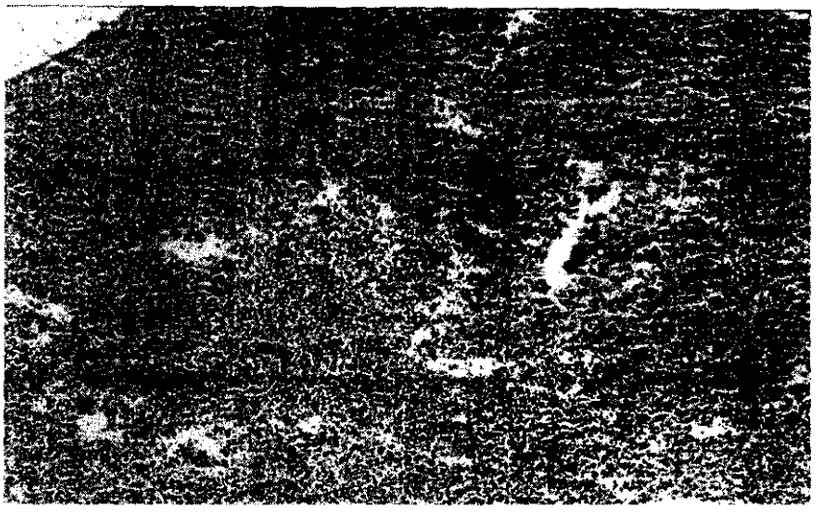

Fig.8: A micrograph showing hyperactivation of melano-macrophage centers also diffuse infiltration with melanophores within the spleenic tissue in the spleeen of *Clarias gariepinus* exposed to 1/10 of LC_{50} of B(a)P for 6 weeks. (H&E, x 300).

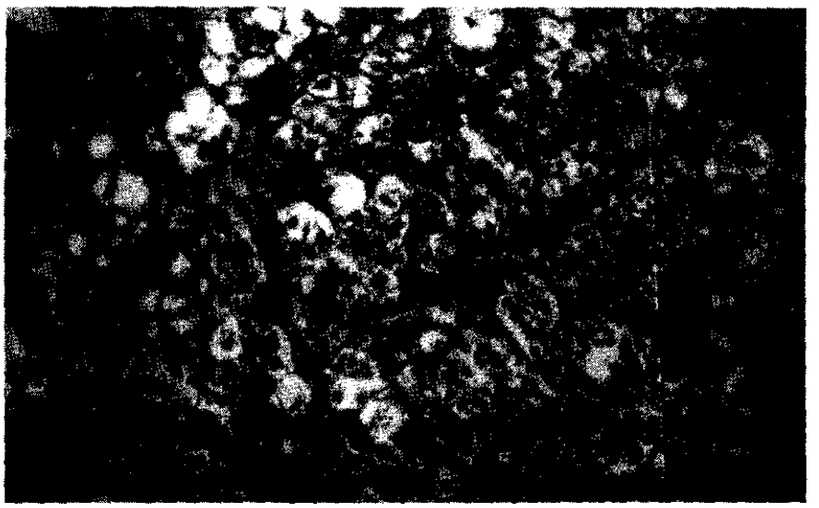

Fig.9: A micrograph showing necrosis of many spermatocytes in the seminiferous tubules in the testis of *Clarias gariepinus* exposed to 1/2 of LC_{50} of B(a)P for 1 week. (H&E, x 400).

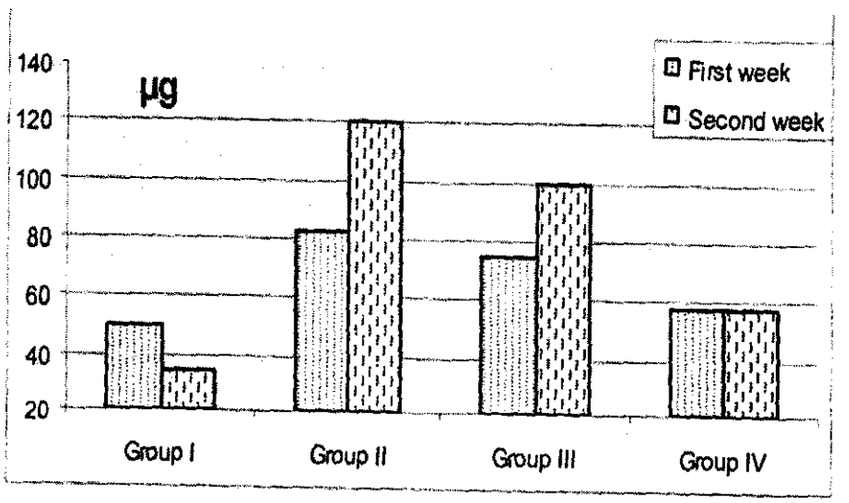

Fig.10:A diagram showing the effect of both B(a)P and probiotic on serum lysozyme after first and second week of exposures where(Group, I):0.1ppm B(a)P for 4w ; (Group, II): 0.1ppm B(a)P+ probiotic,10g/Kg artificial diet for 4w ; (Group, III): probiotic,10g/Kg artificial diet for 4w and (Group IV) acted as a control.

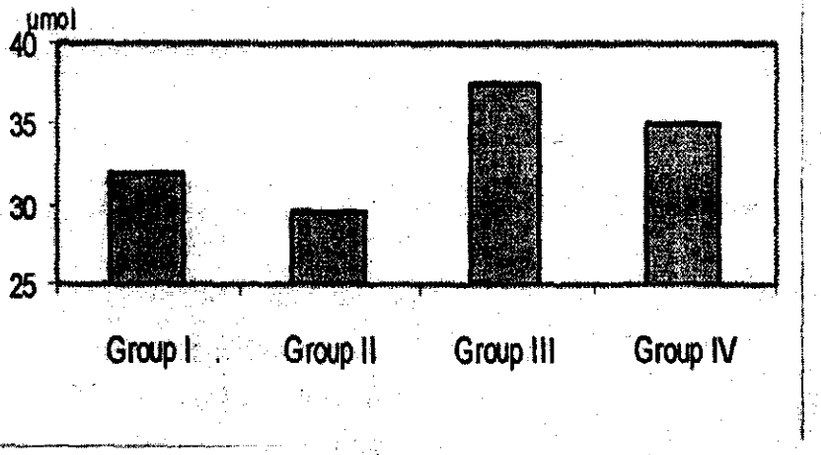

Fig.11:A diagram showing the effect of both B(a)P and probiotic on serum nitric oxide level where(Group, I):0.1ppm B(a)P for 4w ; (Group, II): 0.1ppm B(a)P+ probiotic,10g/Kg artificial diet for 4w ; (Group, III): probiotic,10g/Kg artificial diet for 4w and (Group IV) acted as a control.

Fig. 12: Micronuclei induced by B(a)P exposure in *Clarias gariepinus*.

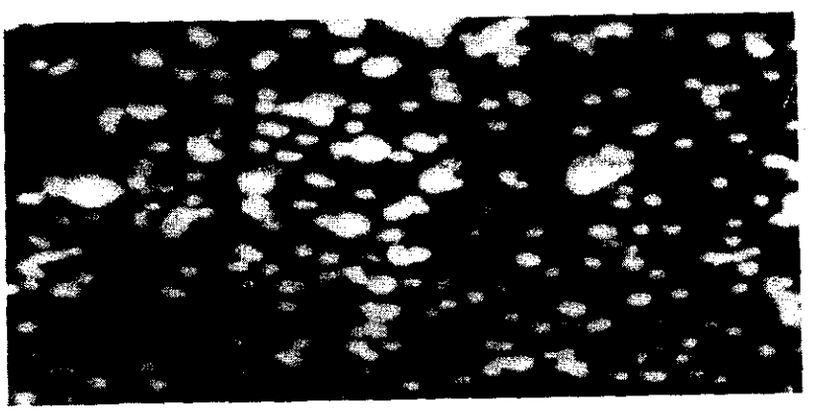

Fig. 13: Comet cells induced by B (a) P exposure of *Clarias gariepinus* Erythrocytes. Arrows show different degrees of DNA breakage and tail lengths.

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البنزوابيرين: تقييم المخاطر والتحديات فى القرموط الإفريقى

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فى هذه الدراسة تم تقييم مدى خطورة البنزوابيرين على أسماك القراميط وكذلك دراسة مدى أهمية إستخدام Diamond-V كابروبيوتك محفز مناعى ضد التأثيرات الضارة لمادة البنزوابيرين من خلال:

١- **دراسات إكلينيكية**: ظهرت أعراض إكينيكية نتيجة تأثير البنزوابيرين فى صورة أعراض عصبية وأعراض تنفسية وتغيرات فى لون الجلد بالإضافة إلى ٢٥٪ ونفوق وبإستخدام البروبيوتيك ظهر تحسن فى الحالة العامة للأسماك وإنخفاض النفوق إلى ١٠٪.

٢- **دراسات هستوباثولوجية**: أثبتت هذه الدراسات العديد من التغيرات التحللية والتليف والأورام فى الأنسجة المختلفة للجسم. بإستخدام البروبيوتك لم يكن هناك تغيراً ملحوظاً من خلال هذه الدراسة على المستوى الهستوباثولوچى.

٣- **دراسات مناعية**: أثبتت هذه الدراسة للبنزوابيرين تأثيرات قمعية مخفضة لليزوزيم، أكسيد النيتروز (No) ، والجلوبيولين وكذلك النسبة التقديرية للبروتين الكلى فى الدم. كذلك وجد أن إستخدام البروبيوتك كان له تأثير إيجابى على إرتفاع تسب هذه البروتينات فى الدم منفرداً، وكذلك تأثير إيجابى إلى حد ما فى حالة إستخدام مع البنزوابيرين.

٤- **دراسات سيتولوجية: تم إستخدام نوعين من التحليلات هى:**

- تحليلات Mn داخل الجسم. وأظهر هذا التحليل زيادة فى عدد النوويات الصغيرة فى حالة إستخدام البنزوابيرين لمدة قصيرة (٧ أيام) وتزداد النسبة كذلك فى حالة التعريض لمدة طويلة (٣٠ يوم). وفى حالة إستخدام البروبيوتك مع البنزوابيرين وجد بالفحص المجهرى إنخفاضاً فى نسبة النوويات مقارنة بالتعريض للبنزوابيرين منفرداً.

ب- تحيلات الـ Comet assay (المذنبات) خارج الجسم. أثبت إستخدام تقنية المذنبات إن للبنزوابيرين تأثيرات خطيرة تتمثل فى تلف المادة النووية L NA وتكسرها وخروجها من النواة، ولقد وجدت علاقة طردية بين نسبة التلف الناتج فى المادة الوراثية وتركيز البنزوابيرين وكذلك طول فترة التعريض.

من هذه الدراسة نستخلص أن مادة البنزوابيرين لها تأثير شديد السمية على القرموط الإفريقى. ينصح بإضافة الـ Diamond-A كابروبيتوتك للأعلاف فى المزارع السمكية لما له من تأثير مقوى للجهاز المناعى وكوقاية للخلايا من تأثير السموم.