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Pharmaceutical effluent induced chromosome aberration in rat bone marrow cells

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ABSTRACT

This paper presents the genotoxic effect of effluent from a pharmaceutical plant in Ota, Ogun state, Nigeria using the rat bone-marrow metaphase chromosome assay. The effluent was examined for some standard physical and chemical properties. Rats were intraperitoneally exposed to 1%, 5%, 10%, 25% and 50% (v/v, effluent/distilled water) concentrations of the effluent for 48 hr. Distilled water and cyclophosphamide (40 mg/kg body weight) were used as the negative and positive controls respectively. The erythroblasts of bone marrow cells examined post treatment show structural chromosomal abnormalities such as translocation, acentrics, breaks, dicentrics and rings. The effluent also induced concentration-dependent significant ($p<0.05$) reduction in mitotic index. Physico-chemical analysis of the tested sample showed the presence of constituents capable of inducing mutation in biological system. The interaction of these constituents with the genetic material in the bone marrow cells caused the observed anomalies. These findings indicate that the tested pharmaceutical effluent is a potent clastogenic and mutagenic agent and suggest potential adverse health risks to exposed living organisms.

Keywords: Pharmaceutical effluent, Rat, Chromosome aberration, Mutagenicity, Clastogenicity.

INTRODUCTION

Recent decades have brought increasing concerns for potential adverse human and ecological health effects resulting from the production, use and disposal of numerous chemicals that offer improvements in industry, agriculture, medical treatment, and common household conveniences [1]. Over the years, there has been a continuous expansion of the pharmaceutical industry, and considerable advances have been made in the discovery of chemotherapeutic drugs that are effective in curing various diseases [2]. Pharmaceutical plants generate a wide variety of wastes during manufacturing, maintenance and housekeeping operations. While maintenance and house keeping activities are similar from one plant to the next, the actual processes used in

pharmaceutical manufacturing vary widely. With this diversity of processes comes a similar diverse set of waste streams. Typical waste streams include spent fermentation broths, process liquors, solvents, equipment wash waters, spilled materials and used processing aids [3]. The disposal of this type of wastes is of environmental concern. Little is known about the extent of environmental transport and ultimate fate of many synthetic organic chemicals after their intended use, particularly hormonally active chemicals [4].

The discovery of a variety of pharmaceuticals in surface, ground and drinking waters is raising concerns about their potential adverse environmental consequences. Minute concentrations of endocrine disruptors, some of which are pharmaceuticals, are having detrimental effects on aquatic species and possibly on human health and development [5]. The available literature on genotoxicity of pharmaceutical effluent is mostly on micro-organism [6-10]. In this study, the mutagenic potential of a pharmaceutical effluent was investigated using the rat bone marrow metaphase chromosome analysis. This is an effective and sensitive short-term *in vivo* bioassay that utilizes cytological damage as an end-point in detecting and screening chemical agents that induce chromosomal damages and rearrangement *in vivo* [11]. *In vivo* cytogenetic methods are preferred to *in vitro* tests because of metabolic activation and detoxification of compounds that occurred in the intact animals [12, 13]. Some of the physico-chemical characteristics of the effluent were also determined according to APHA [14].

MATERIALS AND METHODS

Biological Materials

Male albino rats (*Rattus norvegicus*, 5-6 weeks old, average weight = 75g) were obtained from the animal breeding unit of the Department of Physiology, University of Ibadan. They were acclimatized for 2-3 weeks in in the animal house of the Department of Zoology, University of Ibadan. They were provided with food (Ladokun pelleted feed®) and drinking water *ad libitum.*

Collection of effluent and determination of physical and chemical Parameters

Raw effluent from a pharmaceutical plant in Ota, Ogun state, Nigeria was collected in two 10 L plastic containers at the point of discharge into the environment. The effluent was filtered, pH measured and kept at 4^oC until use. The sample was analyzed for a number of standard physical and chemical parameters including chemical oxygen demand (COD), total dissolved solids (TDS), alkalinity, biochemical oxygen demand (BOD), chlorides, nitrates, ammonia and phosphates according to methods described by APHA [14]. Seven heavy metals namely cadmium (Cd), copper (Cu), chromium (Cr), iron (Fe), zinc (Zn), nickel (Ni) and manganese (Mn) were analyzed in the effluent sample according to standard analytical methods [14, 15]. Briefly, 100 ml of the effluent was digested by heating with concentrated HNO_3 and the volume was reduced to 3-5 ml. This volume was made up to 10 ml with 0.1 N HNO_3. The concentrations of the metals were estimated using an Atomic Absorption Spectrophotometer (Perkin Eelmer E. Analyst, 2000 USA).

Administration of chemicals

Five concentrations: 1, 5, 10, 25 and 50 % (v/v, effluent/distilled water) of the effluent sample were utilized using 3 rats per concentration. Each rat in each group was given a single intraperitoneal (IP) injection of 1.0 ml for 2 consecutive days (48 hr exposure period) [16, 17].

Three rats each were used for the negative (distilled water) and positive (cyclophosphamide 40 mg\kg body weight) controls respectively.

Preparation of bone marrow metaphase chromosome

Each rat/group was injected IP with 1.0 ml of colchicine (3 mg\kg body weight) 2 hr prior to sacrifice at the end of the exposure period. The rats were sacrificed by cervical dislocation and their femoral bones surgically removed. The femurs were immediately put in normal saline, the epiphyses were cut off and the bone marrow was aspirated using 2.2 % (w/v) solution of sodium citrate. The suspension was then centrifuged for 10 minutes at 2000 rpm. The supernatant was decanted and replaced with 0.075 M potassium chloride solution and allowed to stand for 30 minutes. The mixture was centrifuged again at the same speed and time. The supernatant was decanted and replaced with freshly-prepared cold fixative (methanol:glacial acetic acid, 3:1 v/v). This was allowed to stand for 10 minutes after which it was centrifuged for 10 minutes at 2000 rpm. The supernatant was decanted and replaced with fresh fixative. The process of fixing and centrifuging was done thrice. Slides were prepared by dropping the fixed cells from a height of 30-40 cm onto clean, dry, grease-free slides. Finally, the slides were air-dried and stained with 5 % Giemsa (v/v, stock Giemsa stain/distilled water) for 10 minutes. All slides were analyzed blind to treatment at x1000 for chromosomal aberrations. Fifty well spread metaphases were scored per rat for chromosomal aberration and approximately 3000 cells/concentration were analysed for the mitotic index (MI).

Statistical analysis

The Microsoft Excel ® was used for data analysis; and the experimental unit (n) for the analysis was the individual rat. Results are presented as mean ± SE for each concentration. The frequency of chromosomal aberration was computed as the number of aberration per total metaphases at each concentration. The mitotic index (MI) was obtained as the number of metaphases per total cells scored at each concentration. The statistical significance was estimated at 0.05 level of probability using the student's t-test.

RESULTS AND DISCUSSION

Table 1 shows the results of the physical and chemical analysis of the pharmaceutical effluent sample. The sample was dark brown and pH was 6.82. The chloride, nitrate, phosphate and ammonia levels were high; likewise Zn, Mn, Fe and Cu. Figure 1 shows the frequency of the different types of chromosomal aberrations induced in bone marrow cells of rats after exposure to different concentrations of the pharmaceutical effluent. The positive control induced statistically significant ($p<0.05$) number of different types of CA. The aberrations induced by the tested sample were significantly different from the negative control ($p<0.05$, except at the 1 and 5 % concentrations), and these include translocation, ring chromosome, chromatid break, acentric and dicentric chromosomes (Figure 2 a-e). Chromosomes with gaps was observed but were not included in the categories of damaged cells, based on the general opinion [18 -20] that gaps are not good indicators of chromosome damage. The concentration of the sample with the highest number of aberrations was 50% (63% occurrence) while the concentration with the lowest number was 1% (26% occurrence).

Table 1. Physico-chemical analysis of the pharmaceutical effluent assessed for induction of chromosomal aberration in bone marrow cells of rat

Parameters*	Pharmaceutical effluent	FEPA[a]	USEPA[b]
pH	6.82	6-9	6.5-8.5
Colour	Dark brown	-	-
COD[c]	205.86	50	410
TDS[d]	84.00	2000	500
BOD[e]	68.60	50	-
Salinity	82.00	-	-
Alkalinity	110.00	250	20
Chlorides	820.00	-	250
Sulphates	96.80	500	10
Nitrates	28.02	20	10
Phosphates	18.00	5.0	-
Ammonia	98.20	0.01	0.02
Cu	4.7	0.01	1.0
Fe	41.24	0.3	0.3
Cd	ND	0.01	0.005
Al	0.98	-	-
Cr	0.04	0.05	0.10
Mn	0.15	0.05	0.05
Ni	0.005	0.05	-
Zn	8.22	5.0	5.0

**All values are in mg/L except salinity (ppt.) and pH, [a] Federal Environmental Protection Agency (2001) [b] United States Environmental Protection Agency,1989 (www.epa.gov/safewater/mcl:html), [c]COD- Chemical oxygen demand, [d]TDS- Total dissolved solids, [e]BOD- Biochemical oxygen demand, ND- Not detected*

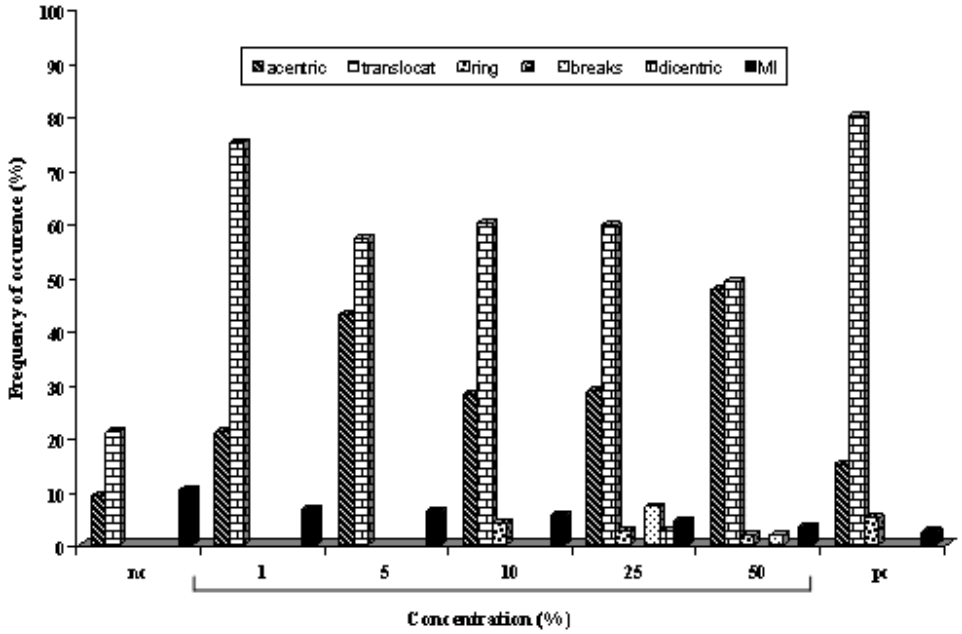

Fig.1. Frequency of occurrence of different types of chromosomal aberrations, and mitotic index in bone marrow cells of rats exposed to the pharmaceutical effluent.
nc: negative control (distilled water), pc: positive control (cyclophosphamide 40 mg\kg body weight)

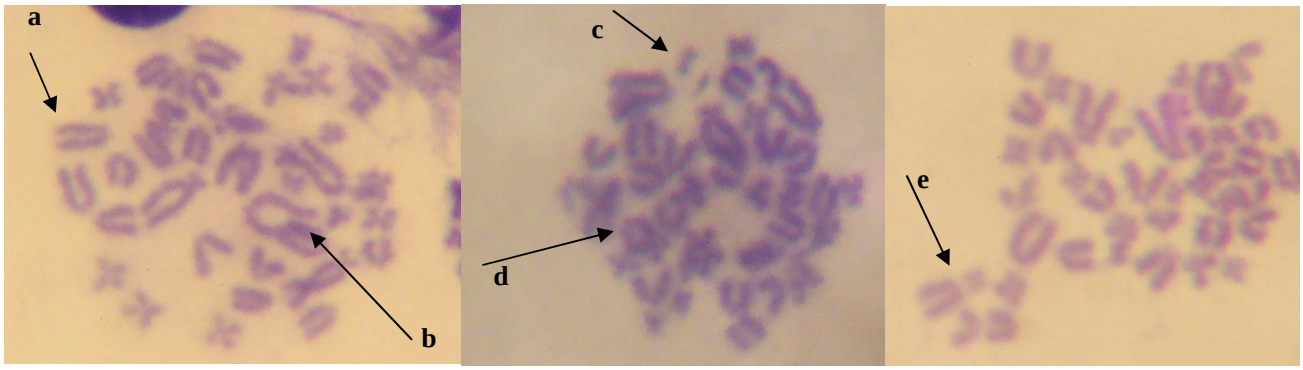

Fig. 2. Chromosomal aberrations (arrowed) induced in bone marrow cells of rats by the Pharmaceutical effluent. (a) acentric, (b) translocation (c) chromatid break, (d) ring chromosome, (e) chromosome break. Magnification-1000x.

The type of aberration that occurred most is translocation (57.9%) with the least being dicentric chromosomes (0.6%). Figure 1 also shows the trend of mitotic index in the analyzed bone marrow cells. Compared with the negative control, the effluent induced a concentration dependent statistically significantly ($p<0.05$, except at a concentration of 1%) reduction in MI.

Most of the heavy metals except Zn, Mn, Fe and Cu were observed at low concentrations when compared with acceptable limits by international regulatory authorities (Table 1). The analysis of these metals was for elemental metals. Because the chemical form of these metals is unknown, it is not possible to estimate the full contribution of these metals to the observed response. In addition, the tested effluent is a complex mixture which contains organics (though not analyzed herein) and other unidentified substances. Interactions (which may include synergy, potentiation, antagonism and additivity) among these chemicals in the mixture with one another as well as with the genetic material in the bone marrow cells of rat led to the present observation. Individually, these metals also have the potential to induce mutations, cancer and other adverse health effects in living cells [21 -28].

A means of detecting *in vivo* genetic activity is to examine mitotically active cells that have been arrested at metaphase for structural changes and re-arrangement of their chromosomes. Chromosome analysis of bone marrow cells *in vivo* has become a standard method for testing for the potential mutagenic effects of viruses, radiation, drugs, and chemical pollutants [29, 30]. The results obtained in this study shows that the tested pharmaceutical effluent induced different types of CA and reduced the number of dividing cells at the tested concentration. A dicentric chromosome is an aberrant chromosome having two centromeres. Apart from the associated loss of chromosomal material, a dicentric chromosome could result in abnormal chromosome behaviour during cell division resulting in faulty transmission of genetic information to daughter cells. Translocation involves the interchange of chromosome segments between two or more homologous and or heterologous chromosomes. Ring chromosome forms when a portion of a chromosome has broken off and formed a circle or ring. They are basically deficiencies and therefore render the carrier liable to the usual consequences of a missing genetic material. An acentric chromosome is an aberrant chromosome that lacks a centromere. A break is a true discontinuity with clearly dislocated fragments and also includes fragments without obvious origins. Acentric fragments and those resulting from breaks almost likely never survive the next

generation and the consequence of this may be grevious due to missing chromosomal material in the next generation.

A possible mechanism for effluent induced chromosomal aberration involves the formation of free radicals, either via auto-oxidation or by enzyme-catalyzed oxidation of organic compounds in effluent. These free radicals could react with lipids and lead to lipid peroxidation of cell membrane in tissues causing the breakage of the DNA chain by oxidating the base in DNA and covalent binding between the product of lipid peroxidation and DNA [31, 32]. They could react with proteins, affect the structures and functions of enzymes, and alter membrane properties [33]. In addition, the free radicals could also attack nucleic acids, especially some spots in purine and pyrimidine, resulting in base substitution and breakage of DNA, and eventually induce mutation [33]. Some of the constituents of the tested sample are considered toxic to drinking water [34]. They have the potential to produce taste and odor problems if allowed to contaminate surface and ground waters. Such contaminated water would be of inferior palatability and may induce an unfavorable physiological reaction in the transient consumer. It is also known that organisms inhabiting areas influenced by effluent discharges can suffer deleterious somatic effects or genetic damage and that people using polluted water could be at higher risk of similar genotoxic effects and cancer development [35 - 37.

Information on CA inducing capacity of pharmaceutical effluents is rare in the broad field of waste water genotoxicity. Only very few studies have reported the genotoxicity and mutagenicity of pharmaceutical wastes [6 -8, 10, 38 - 40]. The present observation corroborates previous findings in *Allium cepa* and mice [41] wherein effluent from a pharmaceutical plant in Lagos state; Nigeria induced CA, micronucleus and abnormal sperm morphology. The presence of chromosomal aberrations in rat bone marrow cells suggests that the tested pharmaceutical effluent is potentially clastogenic and genotoxic. Chromosomal abnormalities are associated with the appearance and/or progression of tumors [42]. It may be plausible to suggest that chemical mixtures such as the tested pharmaceutical effluent may induce chromosome breakages which may consequently lead to cancer. Results obtained in this study indicates that the tested mixtures may be harmful to the environment and human health if it contaminates ground or surface water used for human activities. In conclusion, data obtained in this study suggests that the tested pharmaceutical effluent contained substances capable of inducing DNA damage in rat. Therefore, the direct and indirect exposure to this effluent may cause mutagenic/carcinogenic changes in exposed individuals.

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