Industrial effluents induced abnormal sperm cells in mice (*Mus musculus*)

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Abstract: The *in vivo* genotoxic effects of wastewaters from Agbara industrial estate, Ogun State, Nigeria was investigated using the mouse sperm morphology assay. Two wastewater samples; before treatment (BT) and after treatment (AT), were collected and characterized for some physico-chemical properties in accordance with standard methods. Sperm of mice were examined for morphological abnormalities after 35 days from the first day of exposure to the test samples. Genotoxicity in the mouse was investigated at 5 different concentrations of 1%, 5%, 10%, 20% and 50% of the effluent samples. Tap water and Cyclophosphamide (20 mg/kg bwt) served as negative and positive controls respectively. The samples contained constituents above the permissible limit for the discharge of effluents into the environment. There was concentration-dependent and statistically significant ($p<0.05$) induction of abnormal sperm cells at tested concentrations. Abnormalities observed were believed to be due to the interaction of the effluent constituents with the genetic material in the sperm cells. The results suggest that the tested industrial wastewaters contained chemicals that are potential germ cell mutagens.
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Introduction

One of the major problems facing mankind today is environmental pollution. In developing nations, human population is suffering the effects of pollution caused by increasing urbanization and industrialization (Barberio *et al*., 2009). Pollution is a crucial threat to our environment because it can have an immediate effect on the environment or its interaction after release with moisture or other pollutants in the environment may pose a threat to the survival of mankind. The most severe is related to the disposal of untreated, contaminated and partially treated toxic substances generated by industrial activities. Many hydrological systems in developing regions are getting close to being stressed beyond repair due to industrial wastewater discharges. Effluents from industrial processes are known to contain large amount of synthetic compounds, , most of which are incompletely degradable and potentially harmful (Laws, 1981,Prasad, 2000, Rank et al., 2002) The genotoxic effects of industrial wastes have been examined in microbial (Mathur *et al*., 2006), higher plants (Rank and Nielsen, 1998; Saleem *et al*., 2005), animals (Yang *et al*., 1999; Siddique *et al*., 2008) and human cells *in vitro* (Bakare *et al*., 2007; Garaj-Vrhovac *et al*., 2009). Babatunde and Bakare (2006) investigated the cytotoxic and genotoxic effect of wastewaters from Agbara Industrial estate using the *Allium cepa* assay. The study indicated that the tested effluent samples inhibited root growth and induced chromosomal aberration in the root tip cells of *A. cepa*. From these observations, it was recommended that the potential genotoxic effects of the effluent samples be evaluated in mammalian system. In this study, we investigated the genotoxicity of treated and untreated effluents from Agbara industrial estate, Ogun state, Nigeria using the mouse sperm morphology assay. We also analyzed some of the physico-chemical properties of the effluent samples according to standard methods.

Materials and methods

Agbara industrial estate water treatment plant is located in Agbara town, a border town between Ogun State (longitude 2°30'E and 5 °6', latitude 6 °24'N and 7 °58') and Lagos State (longitude 2 °30' and 4 °22'E, latitude 6 °20'N and 6°40'); in Ado-Odo local Government area of Ogun State, Nigeria. The estate houses about 32 industries and a residential estate occupying about 267 hectares of land. Effluents from the industries and the residential quarters empty into an aerated lagoon system sewage treatment plant that is 250x 600m in size. Treated effluent from this plant is discharged into River Owo, from where it is washed into the Ologe lagoon. Residents around this lagoon are dependent on the river for drinking and for economic activities (Sowunmi, 2001).

Raw effluent (designated as BT) was collected at the point of influence into the plant and treated effluent (designated AT) at the point of discharge into the river using 10 litre plastic containers with screw caps. Grabs of each sample were collected and

pooled to form the stock for each designated sample. The temperature and pH were measured immediately before the samples were transported to the ecology and environmental biology laboratory of the Department of Zoology of the University of Ibadan and stored at 4°c until use.

Sperm morphology assay

This test was carried out according to standards (Wyrobek *et al*., 1983; Bakare *et al*., 2009). Male Swiss albino mice (10-12 weeks old) obtained from the animal breeding unit of the Nigeria Institute of Medical research (NIMR) Lagos, were used for this study. They were kept in a pathogen –free, well ventilated environment of the animal house of the Department of Zoology, University of Ibadan, Nigeria for 2 weeks in order for them to acclimatise. Supply of food and water was uninterrupted since under nourishment is known to affect sperm quality (Krzanowska, 1981). Prior to animal exposure, the wastewater samples were filtered using 2.5-µm filter (Whatman® No. 42) in order to remove sediments, then the supernatant was used in preparing the various test concentrations, however, no tests were conducted for microbial presence. Mice were allotted to 2 test groups (a group correspond to a sample) at random, each group being made up of 5 different concentrations of 1%, 5%, 10%, 20% and 50 % (v/v; effluent: tap water) of the sample. Test animals (6 mice /concentration) were exposed via single intraperitoneal injections of 0.5ml/day/mouse of each concentration for 5 consecutive days. Six mice each injected IP with tap water and cyclophosphamide (Bristol-Myer Squibb® (20 mg/ kg body weight) served as negative and positive controls respectively. The 5-week exposure period from the first day of exposure was considered since it takes 34.5 days for spermatogenesis to complete in mice (Bartke *et al.*, 1974). At 35-day post treatment period, the mice from each group were sacrificed by cervical dislocation and their caudal epididymes were surgically removed and minced in an isotonic medium of normal physiological saline. Sperm smears were prepared on clean grease-free microscope slides after staining with 1% Eosin Y stain for 45 minutes. Slides were air-dried and coded for subsequent microscopic examination at x1000. Six slides were prepared per mouse out of which four were randomly selected for scoring and a total of 600 sperm cells per mouse were assessed for spermhead abnormalities according to the criteria of Wyrobek *et al*. (1983).

Physico-chemical characterization:

The physico- chemical properties of the effluent samples were determined in accordance with standard analytical methods (USEPA, 1996; APHA, 1998). The concentrations of heavy metals namely copper (Cu), Lead (Pb), Manganese (Mn), Chromium (Cr), Magnesium (Mg) and Nickel (Ni) in the effluent sample were estimated after digestion of the effluents with concentrated nitric acid using Buck Scientific® Atomic Absorption Spectrophotometer 205.

The SPSS11.0® statistical package was used to analyse the data. All data are presented as the mean ± standard error. The levels of statistical significance between the negative control and the treated groups was estimated at $p<0.05$ using the student's t-test.

Results:

Table 1 presents the physical and chemical properties of the effluents. BT was acidic while AT was slightly neutral. The values of some of the parameters in the samples were higher when compared with the maximum allowable level for drinking water and that of standard guidelines for uniform effluent discharge limit in Nigeria (FEPA, 1991).

Table 1. Some physico-chemical characteristics of the influent and effluent samples from Agbara industrial estate, Lagos Nigeria

Parameters*	BT	AT	FEPA	USEPA
pH	4.52	6.70	6-9	6.5-8.5
Colour	Milky white	Brown		
BOD[a]	6.94	5.5	50	-
COD[b]	9.4	7.3	-	410
Turbidity	27	23		
Nitrate	4.60	3.68	-	10
Sulphate	7	5	20	250
Phosphate	209.0	200.0		
Ammonia	2.35	33.0	0.01	0.02
Magnesium	1.90	1.72		30
Manganese	2.00	2.20	0.05	0.05
Copper	2.79	2.60	0.3	1.0
Lead	1.90	1.70	0.01	0.015
Chromium	1.95	1.67	0.05	0.10
Nickel	2.55	2.42	0.05	-

* All values are in mg/l except temperature which is in °c and turbidity in *ftu*

[a] Biochemical oxygen demand

[b] Chemical oxygen demand

[c] Federal Environmental Protection Agency (1991) permissible limits for drinking water (Nigeria)

[d] (www.epa.gov/safe/mcl.html)

BT- Effluent before treatment

AT- Effluent after treatment

Table 2 shows the effects of the two effluent samples on mouse sperm morphology. The proportion of sperm abnormalities in the positive control was 33% while the negative control value was 3.13%. Tested concentrations of 1%, 5%, 10% 20% and 50%, the mean abnormalities are 14.80 (± 4.39), 22.8 (±1.93), 26.6(± 4.60), 26.4 (± 7.63) and 25.6 (± 5.91) for the effects of BT and AT effluent samples on sperm head morphology.

Table 2: Effects of BT and AT effluent samples on sperm head morphology

Concentration(%)	BT Total abnormal sperm	%	χ±SE	AT Total abnormal sperm	%	χ±SE
1	73	2.4	14.8±4.39	111	3.7	22.2±3.37
5	111334	3.8	22.8±1.93	126	4.2	25.2±3.14
10	132	4.4	26.6±4.60	114	3.8	22.8±0.80
20	132	4.4	26.4±7.63	182	6.1	36.4±9.06
50	128	4.3	25.6±5.91	199	6.6	39.8±3.25
Total	580	3.9		732	4.8	

This induction was concentration – dependent and statistically significant ($p<0.05$) at tested concentrations when compared to the negative control. Sperm abnormalities scored included sperm with amorphous head, banana-shaped head, nubbed hook, no hook, two tails, pin tail and wrong tail attachment, but no specific abnormality type was observed to be predominant.

Fig 1 shows a comparison of the effects of treated effluents (AT) and untreated effluents on sperm head morphology.

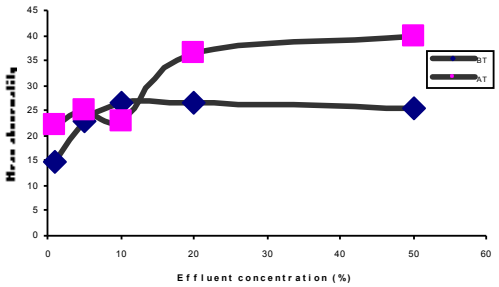

Fig. 1: Effect of influent and effluent from Agbara Industrial Estate, Nigeria on mouse spermhead shape

Discussion:

Investigations into the genetic consequences of chemically induced sperm changes have focused mainly on understanding the genetic basis of induced shape abnormalities in mouse spermatozoa with the belief that such morphological changes may reflect genetic damage in the male germ cell. Results from extensive studies on the genetics of mammalian gametes reveal that the variations in the DNA content of spermatozoa and the gross morphological defects are often polygenetically controlled in a series of complicated synchronized morphological and biochemical steps which, result in nuclei that are normally stable with marked strain-specific structural definitions (Beatty, 1970, Wyrobek *et al*., 1983). Thus, the abnormalities observed in sperm heads probably occur during spermatogenesis. In this study, untreated and treated effluent samples from Agbara industrial estate Nigeria, induced concentration – dependent and statistically significant increase in abnormal sperm cells in mice.

The total percentage abnormalities in the raw effluent (BT) almost approximate that of the treated effluents (AT) (Table 2) but AT at the higher concentrations of 20 and 50 % induced more anomalies than BT, this observation is a paradox as treatment is supposed to reduce toxicity and contamination level, this suggests that some of the effluent's constituents may have acted synergistically with the treatment negatively thereby increasing the effluents' toxicity or that the treatment protocol is generally ineffective. The results indicatesthat the effluent's constituents (had a negative effect on the sperm which had arisen in the exposed spermatogonia. The damage may have taken place at the pre-meiotic stages of spermatogenesis before DNA synthesis (Monesi, 1962). It may also be due to chromosomal aberrations or point mutation that occurs during spermatogenesis. Once sperm head develops completely, its shape and genetic make up is extremely stable. The implication of this is that sperm with an abnormal shape may contain abnormal genetic material (Bruce *et al*., 1974). Although some mistakes do occur naturally in the process of spermatogenesis, the incidence of abnormal sperm heads in unexposed (negative controls) mice is low and ranges between 2-5% depending on age and strain (Krzanowska, 1981). The percentage abnormalities observed in the negative controls herein falls within the stipulated range (Krzanowska, 1981; Wyrobek *et al.*,1983). The constituents of the effluent may have acted singly, additively or synergistically to increase the frequency of naturally occurring mistakes during spermatogenesis. For instance, heavy metals such as Cu, Cr and Mn have been associated with poor semen quality in rodents and man (Telisman *et al*., 2000; Masanyi *et al*., 2004; Kumar *et al*., 2005; Yuyan *et al*., 2008; Wirth *et al*., 2007). This result is consistent with previous reports on induction of genetic damage in the male sex cell of mouse by industrial effluents (Friedman and Staub, 1976; Zhao *et al*., 2007; Bakare *et al.,* 2009; Zhang *et al*., 2010). Toxic effects of treated wastewaters on organismal and reproductive health have also been documented (Colborn and Clement, 1992; Schoenfuss *et al*., 2009).

This finding corroborates previous observations on the tested effluents wherein there was induction of chromosomal aberration and inhibition of root growth in *Allium cepa* at different concentrations of both the influent and effluent samples (Babatunde and Bakare, 2006). With these observations, Agbara Industrial estate effluent could be considered to be toxic and potentially genotoxic. It may also mean that the effluent treatment is not very

effective. This is of immense significance in Nigeria, considering that domestic and industrial wastewaters are discharged into the Nigerian environment without adequate treatment in spite of existing regulations guarding the discharge of effluents into the environment.The fact that the effluent samples induced abnormality in mouse sperm cells suggests that the same could happen in other male animal species especially mammals exposed to it. Spermatozoa with abnormal morphology contain abnormal genetic material and the viability of such sperm cells may be impaired with respect to infertility and fetal congenital abnormality. There should be further health effect studies on the fauna of the water body receiving the effluent from the treatment plant; likewise on man in the surrounding environment since they depend on this water for their domestic and economic activities.

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