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Administration of water samples from Okposi and Uburu Nigerian salt lakes decreased sperm number, sperm motility and sperm morphology in the adult male sprague -dawley rats.

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Abstract: *Polycyclic aromatic hydrocarbon (PAHs) composition of Okposi and Uburu salt lakes and the possible effect of its consumption to some reproductive parameters were determined. The Polycyclic aromatic hydrocarbon (PAHs) compounds composition were determined using gas chromatography coupled with mass spectroscopic equipments. 200 adult male sprague -dawley rats sperm number, sperm morphology and sperm mobility were grouped into eighteen groups of A to Q. Group A to D were given 0.5ml, 1.0ml, 2.0mls and 4.0mls of water from Uburu salt Lake. Group E to H were administered 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg of salt from Uburu salt Lake. Group I to L were given 0.5ml, 1.0ml, 2.0mls and 4.0mls of water from Okposi salt Lake and group M to P were administered 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg of salt from Okposi salt Lake while group Q received demonized water to serve as the control. The experiment lasted for 90 days. At the end of 90 days, the animals were sacrificed. Testes, epididymis and prostate gland were immediately removed and dissected out, cleared from the adhering tissues, blotted dried and weighed individually. Tissues homogenates were prepared and were used to measure the extent of variation in sperm number, sperm morphology and sperm mobility. There was a significant decrease in the testicular weight, epididymal weight and prostate weight in all the treated groups. Our result also indicated a significant reduction in the in the sperm number, sperm morphology and sperm mobility. Chemical analysis of the Lakes showed a significant level of some polycyclic aromatic hydrocarbons. No polychlorinated biphenyls were found to be present in both lakes. Result shows that consumption of Okposi and Uburu salt lake unprocessed water and salt samples could be dangerous to health and might lead to reproductive function impairment.*

Key words: *Reproductive toxicity, polycyclic aromatic hydrocarbons, sperm number, sperm mobility, sperm morphology , salt lakes and fertility.*

Lakes are products of volcanic, glacial, tectonic and river activities which leaves depressions and cavities on land surfaces (Charles *et al.,* 1999). A salt lake is a land locked body of water which has a concentration of salts (mostly sodium chloride) and other minerals significantly higher than most lakes (often defined as 3g salt per liter).Salt lakes have been shown to contain metallic and non-metallic ions, such as calcium, cadmium, lead, magnesium, mercury, manganese, fluorine, bromine, sulfate etc, in addition to sodium and chlorine (Agbafor *et al.,* 2010). The physicochemical properties such as temperature ,salinity, pH, carbonate, phosphates, nitrogen and organic compounds has also been shown to be varying at values higher than the World Health Organization's standard for drinking water (Akubugwo *et al.,* 2007). Indeed, a salt lake is a complex solution of minerals and decayed biological matter that results from life in the lake (*Agbafor et al*., 2010)

The chemical constituent of lakes originates from either natural processes (erosions and weathering of coastal materials) or anthropogenic sources (domestic, industrial and agricultural practices) (Charles *et al*., 1999). There is growing concern about contamination of surface water and lakes giving that most of them are used as drinking water source and for domestic purposes. There are a lot of toxicants found in waters, sediments and biota especially persistent organic pollutants(POPs) such as polychlorinated biphenyls(PCBs), organochlorinated pesticides(OCPs) and polycyclic aromatic hydrocarbons(PAHs)(Liu *et al.,* 2000; Liu *et al*., 2003) which could be accumulated and biomagnified in the food chain. Okposi and Uburu salt lakes are located in Ohaozara Local Government Area of Ebonyi State Nigeria .The lakes serve as salt (obtained after heating lake water to dryness) and water source for most domestic purposes of the inhabitant of the communities who are mainly farmers. Akubugwo *et al., (2007*) have reported the presence of metallic and non-metallic ions in the lakes. Cardiovascular toxicity has also been reported (Agbafor *et al.,* 2011). These toxic effects have been attributed to the chemical constituent of the lakes (Agbafor *et al*., l 2011)

The World Health Organization defines infertility as the inability of a couple to achieve conception or bring a pregnancy to term after 1 year or more of regular, unprotected sexual intercourse. Approximately 30 %

of infertilities are due to a male factor. Several conditions can interfere with spermatogenesis and reduce sperm quality and production. Infertility is a major clinical concern, affecting 15% of all reproductive-aged couples, and male factors, including decreased semen quality, are responsible for 25% of these cases. Currently, the etiology of suboptimal semen quality is poorly understood, and many physiological, environmental, and genetic factors, including oxidative stress have been implicated (WHO. (1995). Exposure to insecticide, heavy metals and organochlorinated compounds has been associated in animals and humans with occurrence of spontaneous abortion, low birth weight, birth defects, and change in male: female sex ratio of offspring, inhibition of spermatogenesis and ovogenesis, destruction of seminiferous epithelium and hydrocels resulting to reduction in fertility (Saravu et al., 2007).

Okposi and Uburu salt lakes are one of the most important lakes used as source of water for domestic use and as cooking salt when heated to dryness. There are a large number of farms and cities that are potential source of contamination to these lakes. Carbon tetrachloride is a possible carcinogen that also causes acute effects to liver, kidneys, cardiovascular systems and reproductive impairment (Mari et al., 1991).. The spectrum of possible hepatic effects in animals is broad and includes macrosomal enzyme induction, liver enlargement; increased serum levels of liver enzymes and lipids, and histopathological alterations that progress to fatty and necrotic lesions and tumors

3.2 Methods.

Collection of samples.

The samples used for the study were collected in the month of March 2011 at the peak of dry season. The bottles used for sample collection were thoroughly washed with deionized water. The lakes were divided into transact of North, South, East and West about an epicenter. Four samples were collected randomly from each transact and pooled together to obtain a unity sample that was used for the study according to the method of (Agbafor *et al*., 2011)

Salt sample Collection and preparation.

Five salt samples were obtained from the local people and crushed together to get a homogenous unity sample. A stork solution of 400mg/ml was prepared by dissolving 40,000mg of salt in 100mls of deionized water.

Collection of Animals.

400 male bred Sprague-Dawley rats weighing 170-200g obtained from Manchester school of veterinary institute were used in the study. The animals were housed in a centralized animal care facility maintained at 22 to 25^{o}C with a relative humidity of 76 ± 5%. Standard pelleted food and deionized water were provided for the animals' ad libitium.

Administration of Samples

Salt samples from Okposi and Uburu salt lakes was dissolved in deionized water and was termed sample A and A_1 respectively. The lake raw water samples from Okposi and Uburu salt lake was termed sample B and B_1 respectively. The animal was grouped into 20 groups with each group containing five rats. 50, 100, 200 and 400mg/kg body weight of sample A was administered to group C, D, E and F respectively while group G was given deionized water only to serve as the control group. 50, 100, 200 and 400mg/kg body weight of sample A_1 was administered to group H, I, J and K respectively. 0.5, 1.0, 2.0 and 4.0ml/kg body weight of sample B was administered to group L, M, N, and O respectively. 0.5, 1.0, 2.0 and 4.0ml/kg body weight of sample B_1 was administered to group P, Q, R and S respectively.

Collection of Samples from the animals

The experiment was performed in three batches and in each batch, administration lasted for two months. After the end of two months administration, the animals were sacrificed. Reproductive tissues such as the testes, seminal vesicles, epididymis and prostate gland were removed immediately, cleared from the adhering tissues, blotted dried and weighed individually. Semen samples were obtained from the epididymis and biochemical analysis was carried out in each batch. Administration was done orally.

Quantification of polycyclic aromatic hydrocarbons

Concentrations of PAHs were measured by use of an HP6890 GC/5973MSD (Agilent US) and HP-5MS capillary columns (30m x 0.25mm id x 0.25μm film thickness). The carrier gases were helium and nitrogen for MSD and μECD respectively. The temperature program for PAHs was 40^{o}C for 4minutes, rising to 320^{o}C at 8^{o}C/min and held for 5min. The GC injector and μECD was held at 250^{o}C and 280^{o}C respectively.

Injector and interface temperature for GC/MSD were held at 270^{o}C and 250^{o}C respectively. The method blank contained no detectable amount of target analytes. The detection limits were 0.01mg/l for PAHs

Preparation of homogenate: A fraction of the right testicles and epididymis of each animal was collected for biochemical assays and homogenates were prepared. After perfusion in 0.9% saline, testes and epididymis were homogenized in 0.2M sodium phosphates, pH 6.25 buffer (1:20, w/v) in a potter-elvehjem homogenizer fitted with a Teflon pestle. Homogenates were centrifuged at 10,000xg for 1hr and the supernatants were obtained. The supernatants were stored at -20^{o}C and utilized for biochemical analysis

Collection of epididymal sperm: The epididymides were separated carefully from the right testis. The epididymis was divided into 3 segments; head, body and tail. The epididymal tail was trimmed with scissors and placed in 1.0ml of 0.1M phosphate buffer of pH 7.4. It was then vigorously shaken for homogeneity and dispersal of sperm cells. Semen samples were assessed for number, motility and gross morphology (Mehran *et al*., 2011).

Determinations of testicular cholesterol

Total cholesterol assay kits (Cell Technology Inc, CA94043 USA) were used.

The assay principle was based on an enzyme-coupled reaction that detects both free cholesterol and cholesterol esters. Cholesterol esters are hydrolyzed by cholesterol esterase into cholesterol, which is then oxidized by cholesterol oxidase to yield hydrogen peroxide and cholest-4-en-3-one (ketones). The hydrogen peroxide then reacts with the cholesterol probe (detection reagents) in a 1:1 stoichiometry to produce the stable fluorescent product. The absorbance was read at 570nm according to the kit protocol.

Evaluation of sperm numbers/counts

Epididymal sperm count was performed using the method of (Seed *et al*., 1996) . Briefly, sperm counts were visually determined in a counting chamber by microscopy. 1 in 20 dilutions were made using bicarbonate formalin diluting fluid to mix properly. The improved neubauer counting chamber was loaded with well mixed semen suspension and allowed to stand for 3 - 5 minutes for spermatozoa to settle. The sperm count was made by counting the four corners square and the central large square of the Neubauer counting chamber (improved) and the result was expressed as 10^6 per sperm dilution

Calculation for sperm count

Total number of sperm cells counted in 5 small squares = n
Multiplication factors 50,000 dilution factors = 20
I.e. sperm count per ml = n x50, 000 x 20 x 3
Where 3 is the number of ml of normal saline used in meshing the semen
Normal range of total sperm count = 20 million.

Determination of sperm morphology

A drop of sperm suspension was smeared onto a slide to obtain a uniform smear. After the smear were air dried and stained with Liu's stain (Handsel Technologies Inc, Taipei, Taiwan) the slide were washed in water. While it was still wet, it was fixed with 95% v/v ethanol for 5 – 10 minutes and allowed to air dry. It was then washed with sodium bicarbonate formalin solution to remove mucus. The smear was then rinsed with several changes of water and covered with dilute (1 in 20) Carbon fuschin and allowed to stain for 3 minutes.

The stain was washed off using water and counter stain with 1 in 20 Loffler's methylene blue for 2 minutes. The stain was then washed off Using water and allowed to air dry. And then observed under a microscope for changes in sperm morphology according to (Seed *et al*., 1996). The criteria chosen for head abnormality were; no hook, excessive hook, amorphous, pin, short head, big head and banana head. Coiled flagellum, bent flagellum, bent flagellum tip were recorded as abnormal tailed sperm while those that have oval head with long tail were regarded as normal sperm. The results are reported in percentage i.e. the overall abnormal form.

Sperm motility determination

A drop of well mixed liquefied semen was placed on a slide and covered with a cover glass. The sperm motility as determined by counting both motile and non-motile sperms in at least 10 separate and randomly selected fields using x 10 objective lens microscope to ensure uniform distribution of spermatozoa. The results were expressed as percent motility. The count was repeated three times for each sample to minimize error.

$$\text{Percentage of mobile spermatozoa} = \frac{\text{number of mobile spermatozoa}}{\text{Total number of counted spermatozoa}} \times 100\%$$

I. Results

Table: 1 Compilation of the concentration of polycyclic aromatic hydrocarbons (PAHs) in Okposi and Uburu salt lakes water

PAHs	Sample B Okposi salt lake (mg/l)	Sample B1 Uburu salt lake (mg/l)	NAFDAC (mg/l)
Phthalates	n.d	n.d	0.07
Phenanthrene	0.05	0.06	0.07
Fluoranthene	0.09	0.20	0.07
Benzo(a)pyrene	n.d	0.01	0.07
Benzo(b)fluoroanthene	n.d	n.d	0.07
Coronene	n.d	n.d	0.07
Benzo(ghI)perylene	n.d	n.d	0.07
Pyrene	0.04	0.05	0.07
Benzpo(e)pyrene	0.17	0.24	0.07
Chrysene	n.d	n.d	0.07
Fluorene	0.03	0.05	0.07
Dibenzo(a,h)anthracene	n.d	n.d	0.07
Benzo(I)fluroanthene	n.d	n.d	0.07
Naphthalene	0.76	0.02	0.07
Anthracene	0.08	0.52	0.07
Fluoranthane	0.03	n.d	0.07

n.d = not detected. NAFDAC maximum permissible limit=0.007mg/L.

Table 2: Changes in biochemical parameters of adult Sprague- Dawley rats administered with water from Okposi salt lake.

Parameter	Control	0.5ml/kg	1.0ml/kg	2.0ml/kg	4.0ml/kg
Initial b.wt (g)	198.2±5.8	197.0±4.2	197.8±5.2	196.6±4.9*	197.0±5.8*
Final b.wt(g)	197.2±7.1	194.0±4.4	193.1±5.6	189.5±7.2*	189.04±5.1*
Body weight ratio	0.0079±0.002	0.0049±0.003*	0.0028±0.001*	0.0026±0.0012*	0.0021±0.0010*
Sperm number (x 10^6)	140.42±4.88	129.88±4.62*	112.20±4.02*	98.22±3.80*	82.19±2.80*
Sperm motility(%)	97.2±4.45	95.21±4.42	90.42±4.03*	82.40±3.7*	76.62±2.8*
Sperm morphology (%)	30.0±0.96	26.4±0.82	20.68±0.52*	18.8±0.49*	17.00±0.43*

Values are means ± SD. Values bearing the superscripts * are significantly different from control. n=5

Table 3: Changes in biochemical parameters of adult Sprague- Dawley rats administered with water from Uburu salt lake.

Parameters	Control	0.5ml/kg	1.0ml/kg	2.0ml/kg	4.0ml/kg
Initial b.wt (g)	198.8±4.2	197.0±3.9	195.2±3.3	194.0±3.1	192.0±2.9
Final b.wt (g)	198.8±3.4	189.6±3.6*	190.0±3.0*	184.2±2.9*	180.0±3.1*
Testis wt (mg/100g b.wt)	1321±18.9	1027±31.0*	1237±40.25*	974.0±30.21*	940.0±20.11*
Prostate (mg/100g b.wt)	297.4±12.32	209.0±11.8*	205.2±10.6*	203.0±9.8*	200.2±9.0*
Epididymis (mg/100g.b.wt)	399.7±20.0	260.3±7.8*	261.32±6.8*	258.3±4.8*	250.2±4.6*
Epididymal sperm number (x 10^6)	142.68±5.97	120.68±5.29*	106.6±5.02*	90.49±4.9*	78.40±3.4*
Epididymal sperm motility (%)	99.4±7.72	94.3±7.4*	86.3±6.9*	78.2±5.8*	80.12±5.9*
Epididymal sperm morphology(%)	40.2±0.94	34.1±.89*	28.8±0.78*	22.24±0.67*	18.88±0.32*

Data are means ± SD. Value bearing * are significantly different from the control

Polycyclic aromatic hydrocarbons present in Okposi and Uburu salt lake

Most literature report on physicochemical properties and toxicity effect of Okposi and Uburu salt lakes has been on metal and non metal composition (Akubugwo et al., 2007;Agbafor et al., 2008) and on hepatotoxicity, cardiovascular and renal function impairment (Agbafor et al., 2011) . Not much, if any has been reported on persistent organic pollutant composition and possible reproductive effect of Okposi and Uburu salt lakes. This work was set at determining the composition of polycyclic aromatic hydrocabons pollutant present in these lakes and their effect on the reproductive functions.

Concentrations of PAHs were measured in Okposi and Uburu salt lakes in Ohaozara local Government area of Ebonyi State. Only very few PAHs of the sixteen World Health Organization priority PAHs were observed at quantifiable concentrations (Table 1). The total concentration of PAHs with 3 and 4 rings such as naphthalene, Phenanthrene and fluroanthene were detected at greater concentrations than that of PAHs containing 5 to 6 rings. The concentrations of most of the detected PAHs compounds were less than the water quality limit set by the WHO and NAFDAC which is 0.07mg/L. Sources of PAHs can be assessed by use of ratios of concentrations of individual PAHs (Yunker et al., 2002; Budzinski et al., 1997). The ratios of Phe/Ant within the two ring group of PAHs and Fl/Pyr within the four rings group of PAHs were used to differentiate

among sources. A Phe/Ant ratio >15 suggest petrogenic sources and Phe/Ant ratios of <10 are suggestive of pyrogenic sources. The Fl/Pyr ratio of 0.6 which is < 10 observed in this study indicates that PAHs originated from pyrogenic sources. Petrogenic PAHs generally originate from the leakage of crude oil and the refined products such as gasoline (Yunker et al., 2002). Pyrogenic PAHs originate primarily from combustion especially of fossil fuels.

Effect of both lakes on body weight and organ weight

In the in vivo studies, epididymal cell and testicular cells of adult male rats were chosen to assess the reproductive toxicity of the treatment of salt and water from these lakes which have been proven to contain some organic compounds based on this research and metal including non metals and heavy metals. The testes and epididymis contains sertoli cells, spermatogenic cells and leydig cells. Sertoli cells are one of the most important somatic cells in the reproductive organ (Karl and Capel, 1998). Spermatogenic cells are precursor cells, sperm cells (Reyes et al., 1997) and leydig cells play a role of nutrition, support and mediate transfer (Ewing and Zirkin, 1983).

Administration of a chemical compounds containing metal, non metals and organic compounds may significantly alter the concentrations and metabolic transformation of biomolecules as well as the histoarchitecture of several organs of the animal which may lead to structural and functional dysfunctions. This effects which could be mild or severe may be beneficial or deleterious at the subcellular, cellular and organ levels. In the present instance, the lake's products adversely affected the normal functioning of the rat testes as evidenced by the biochemical and reproductive cell changes in this study.

An increase in the organ-body weight ratio may either indicate inflammation or an increase in the secretory ability of the organ while reduction in the value of organ-body weight ratio may imply cellular constriction. Computed organ body weight ratios therefore can be used to indicate organ swelling, atrophy or hypertrophy. In the present study testes-body weight ratios decreased significantly ($p<0.05$) as shown in our result . There was also significant decrease in body weights and organ weights ($p<0.05$) of the treated rats compared to controls. Therefore, the reduction in the body weights, organ weights and reduction in testes-body weight ratios by the salt and water from both lakes in this study may suggest atrophy, reduced tubule size, spermatogenic arrest and or inhibition of steroid biosynthesis in the leydig cells. This decrease in weight of the animals given salt and water from these lake is in agreement with work of (Sarker et al., 2003; Waalkes et al., 2003) who observed reduction in the weight of the accessory sex organs of animal given NaAsO2. The same thing happen to animals given NaF, Cd and animals administered with Chlorpyrifos and Profenofos pesticides as observed in the work of (Yu et al., 2007). It is also an indication of impairment at testicular, pituitary or hypothalamic level and will adversely affect the normal functioning of the testes.

Effects on sperm parameters

The number of sperm in the caudal epididymis was significantly decreased in animals administered with Okposi and Uburu salt lake samples especially for as the dose and period of exposure increases (table 1 and 2). Progressively, sperm motility decreased significantly ($p<0.05$) in the administered groups compared with the control groups. Sperm morphological examination in the sturdy revealed significant increase in the number of abnormal sperm. Saradha and Mathur, (2006) reported that a decreased density of epididymal sperm could be correlated with testicular spermatogenic arrest and fragment of sertoli cells. In the present study, Okposi and Uburu salt lake samples 'administration resulted in a significant decline in epididymal sperm count, sperm motility and there were substantial abnormalities in the sperm morphology. This may be as a result of androgen deprivation accelerating sperm transition and thus affecting the process of epididymal sperm maturation. Rajeswary et al., (2007) indicated that impaired testes leydig cells could not normally secrete testosterone resulting in the decline of sperm counts. More so, the body weight as well as testis and epididymal weight of treated animals showed significant decrease ($p<0.05$). Most of the testicular biochemical parameters were also significantly decreased compared to their sham controls. Finally, testicular histology confirmed that these lakes samples actually disrupt the activity and functions of the testis by affecting the above discussed biochemical parameters. The present study therefore, demonstrated that sub-chronic administration of these samples reduced sperm number, sperm motility, sperm morphology and some biochemical parameters significantly which indicates an interference with spermatogenesis. Hence, these lakes water and salt therefore needs to be properly treated and refined before its consumption. Otherwise, it could be very toxic to the body when consumed constantly and at high concentration.

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