

*Full Length Research Paper*

## Histopathological and hormonal disrupting effects of Escravos crude oil on the ovary of Chinchilla rabbits

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Crude oil is found in water and soil due to pollution resulting from oil exploration and exploitation. It is used as traditional medicine in some countries, especially among rural dwellers in the south-south and south-eastern Nigeria. The aim of this study was to investigate the effects of Escravos crude oil on serum cholesterol, estradiol and progesterone in the ovary of Chinchilla rabbits. A total of thirty female Chinchilla rabbits of age twelve to fourteen weeks and weighing 1.2 to 1.45 kg were used. Crude oil was orally given at the dose of 15, 20, 25 and 30 mg/kg body weight, corresponding to groups B, C, D and E, respectively for 28 days while group A was the Control. The results showed a significant increase in serum levels of estradiol, cholesterol and ovary weight ( $p < 0.05$ ) while a significant decrease in serum level of progesterone ( $p < 0.05$ ) was observed. The histological findings include: ovarian cysts, fibrosis, marked lymphocytic infiltrations and hydropic cells. Therefore, Escravos crude oil could be considered as a potential endocrine disruptor which can affect the tissue architecture and the endocrine functions of the ovary.

**Key words:** Chinchilla rabbits, Escravos crude oil, estradiol, fibrosis, hydropic cells, progesterone, ovarian cysts, total cholesterol.

### INTRODUCTION

Oil spills are a common event in Nigeria and occurs due to a number of causes including: corrosion of pipelines and tankers (accounting for 50% of all spills), sabotage (28%) and oil production operations (21%), with 1% of the spills being accounted for by inadequate or non-functional production equipment. The largest contributor to the oil spill in total, besides corrosion of pipes and tanks, is the rupturing or leaking of production infrastructures that are described as "very old and lack regular inspection and maintenance" (Nwilo and Badejo, 2001). Between 9 and 13 million barrels have been spilled into the Niger delta since 1958 (Baird, 2010).

Within some Nigerian rural population, crude oil is orally ingested for medicinal purposes. It is claimed to be an antidote to poisoning and a cure for various gastrointestinal disturbances (Eyong et al., 2004). According to Dede et al. (2002), cases of misuse of this substance by individuals have been reported, as it is known to be used liberally by some of the indigenes who believe that it can repel witches when applied topically or given orally to afflicted individuals, while other countries such as Kenya, Tanzania, Zimbabwe, Ghana and Tunisia depend on crude oil for unorthodox treatment of ailments such as stomach ache, diarrhoea, respiratory distress

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and convulsion. The impact of crude oil spillage and discharge on the ecosystem as a result of oil exploration activities is an obvious problem of environmental concern (Otitoju and Onwurah, 2007; Ovuru et al., 2004). The Shell Petroleum Development Company (SPDC) since 1989 recorded an average of 221 spills per year in its operational area, involving 7,350 barrels annually (SPDC Nigeria Brief, May 1995:3). From 1976 to 1996, a total of 4,647 oil spill incidences spilling approximately 2,369,470 barrels of oil into the environment of which 1,820,410.5 (77%) were not recovered. Most of these oil spill incidences in the Niger Delta occur on land, swamp and the offshore environment (Nwilo and Badejo, 2005a, 2004; Twumasi and Merem, 2006).

Effects of petroleum hydrocarbons on reproductive and development processes can include interference of hydrocarbon derivatives with hormone synthesis (Truscott et al., 1983) and an increase in incidence of developmental abnormalities (Hawkes and Stehr, 1982). Polycyclic aromatic hydrocarbons (PAHs) found in Nigerian crude oil(s) such as the Bonny light crude oil act as estrogenic endocrine disruptors (EED) and can cause disruption on both the reproductive tract and endocrine organs (Zhang and Qiao, 2004). PAHs, the major constituents of crude oil, have also been described as endocrine disruptors in fish, especially as modulators of steroidogenesis (Evanson and Van der kraak, 2001). Benzene arene oxide produces destructive and mutagenic effects on various organ systems of test group animals and is implicated in the etiology of cancer (Nwankwoala and Zuworitin, 2002).

Commonly reported effects of acute exposure to crude oil through inhalation or ingestion include: difficulty in breathing, headaches, nausea, confusion and other central nervous system effects (Akpofure et al., 2000). Chronic exposure of animals to crude oil produces signs and symptoms of toxicity involving the central nervous system (Onuoha and Nwadukwe, 1990), the reproductive system (Nte et al., 1997) as well as genotoxic (Kalf et al., 1987; John et al., 1996). Aslani et al. (2000) reported bloody stools, coughing, constipation, infertility and sudden death in female goats exposed to West Texas intermediate crude oil, while studies conducted by Igwebuike et al. (2007) revealed that exposure of male rats to Nigerian Qua-iboe brent resulted in reduced packed cell volume, increased total leukocytes count and reduced cauda epididymal sperm reserves.

Cholesterol is a lipid synthesized by virtually all cells, especially the liver. It functions as structural component of membranes, precursors of bile salts, steroid hormones and vitamin D (Morrissett, 2006). Estradiol is a steroid hormone; it stimulates an increase in the size of the fallopian tubes, uterus, vagina and ovaries. Its deficiency is characterized by underdevelopment of the female reproductive organs. Progesterone is a steroid hormone.

It reduces the frequency and intensity of uterine contractions and thus helps in preventing the expulsion of the implanted ovum. Its deficiency may cause expulsion of implanted ovum. Defective synthesis of the steroid hormones produced by the adrenal cortex and the gonads can have profound effects on reproduction, human development and homeostasis. The aim of this study was to investigate the effect of Escravos crude oil on serum cholesterol, estradiol, progesterone concentrations and the weight and architecture of the ovary.

## MATERIALS AND METHODS

### Test sample

The Escravos blend crude oil (with reference number 863) used in this study was provided by Warri Refining and Petrochemical Company Effurun, Delta State. The crude oil was exposed to sunlight in shallow pans (25 cm × 25 cm × 5 cm) for 24 h at the site of the project to allow the extremely light and volatile fractions to evaporate, leaving behind the stable components. This product simulates the naturally occurring condition following spillage (Neff et al., 2000).

### Animals/experimental design

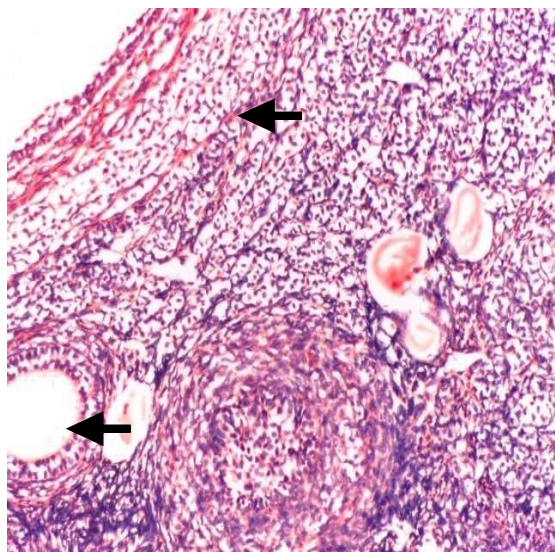
A total of 30 female Chinchilla rabbits aged 12 to 14 weeks and weighing 1.2 to 1.45 kg was obtained from the Faculty of Agriculture, Ebonyi State University Abakaliki (EBSU). The animals were examined, treated for ectoparasites using Ivermectin (Hebei New Century Pharmaceutical Co. Ltd) by a veterinarian and allowed to acclimatize for two weeks at the Animal House of the College of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus. The rabbits were randomly divided into five groups containing 6 female rabbits each. The research plan consisted of four groups designated Group A (control), B, C, D and E. Group B to E were orally given a sub-lethal dose of 15, 20, 25 and 30 mg/kg body of the Escravos crude oil, respectively with due consideration of their body weight (those with greater body weight have their dose divided into two; one in the morning one at night). The different doses of the liquid Escravos crude oil were measured in weight on an electronic weighing balance and given orally (oral gavage) for 28 days.

### Animal treatment

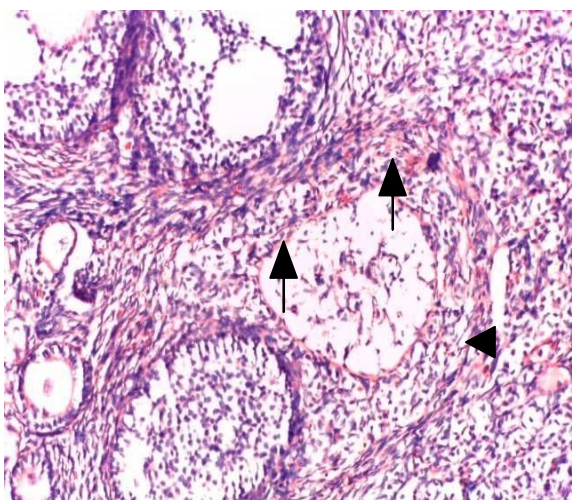
Overnight, prior to exposure, the animals (rabbits) were starved of solid food and their body weights were taken weekly and for the duration of the study to check for weight loss or gain which is associated with toxicity. The rabbits were fed vital grower pellets and water *ad libitum* for 28 days.

### Sample collection, organ harvest and histology

On the 29th day morning, animals were anaesthetized using cotton wool damped in chloroform with due consideration of their body weights. The blood samples, obtained by marginal ear vein puncture, were drawn into tubes using 22 gauge sterile needles. For biochemical analyses, blood samples collected into plain test



**Figure 1.** Group A (control): Ovarian section with a section of a developed (arrow at the top) and developing follicles (arrow head). Stained by H&E Technique.  $\times 200$



**Figure 2.** Group B (15 mg/kg): Ovarian section with interlacing bundles of closely packed fibrocystic cell type with elongated spindle-shaped basophilic nuclei (arrows). The follicles are enlarged and cystic (arrow head). Stained by H&E Technique.  $\times 200$

tubes were centrifuged (Rotofix 32®-Hettich) at 3000 g for 10 min; the serum was collected and kept at  $-20^{\circ}\text{C}$  until analysis. Animals were sacrificed and both ovaries were excised, trimmed of all fat, blotted dry to remove traces of blood and weighed using an electronic weighing balance (using 210/0.1 mg digital balance ESJ-210-4). The excised ovaries were fixed in 10% formal saline processed through paraffin wax. Ovary slices of  $3\ \mu\text{m}$  thickness were stained using Haematoxylin and Eosin (H&E) staining

Technique (Awvioro, 2002) and photomicrograph of the stained tissue sections were taken for documentation. The processing of the ovary was made at Histopathology Unit in the Nnamdi Azikiwe University Teaching Hospital, Nnewi, Anambra State, Nigeria.

### Biochemical analysis

Serum concentrations of estradiol (E2) and progesterone were estimated using the Microplate enzyme immunoassay as described by Radwanska (1976) using a kit from Monobind Inc., USA, and total cholesterol was estimated using the enzymatic end point kit from Randox Laboratories, United Kingdom. These biochemical analyses were done using enzyme linked immunosorbent assay (ELISA) machine, MR 96 USA, and spectrophotometer. The biochemical analyses were carried out using the facilities of Reene Laboratories Onitsha and Immaculate Hospital Nkpor, Anambra State.

### Statistical analyses

Mean values  $\pm$  standard deviation (SD) of the sex hormones, cholesterol, body and ovary weights were taken for analysis. The data was tested for homogeneity of variance and significantly different results were established by one-way analysis of variance (ANOVA) using the statistical package for social sciences (SPSS) software application (version 16). The multiple comparisons were made using the Post hoc test. The accepted level of significance was set at  $p < 0.05$ . The Pearson's correlation was made to compare the blood levels of sex hormones and the accepted level of significance set at 0.01.

## RESULTS

### Behavioural effect

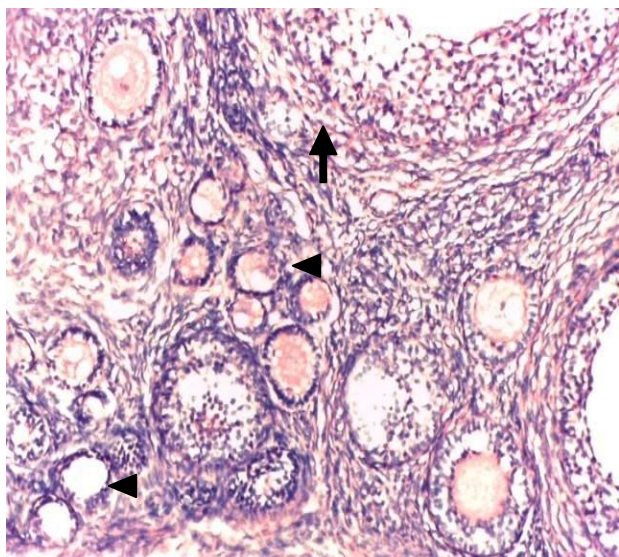
After two days of the crude oil administration, the animals in the treated groups D and E became restless. The latter was followed by loss of appetite and decreased locomotion. Soon after the tenth day, they regained their appetite.

### Biochemical findings

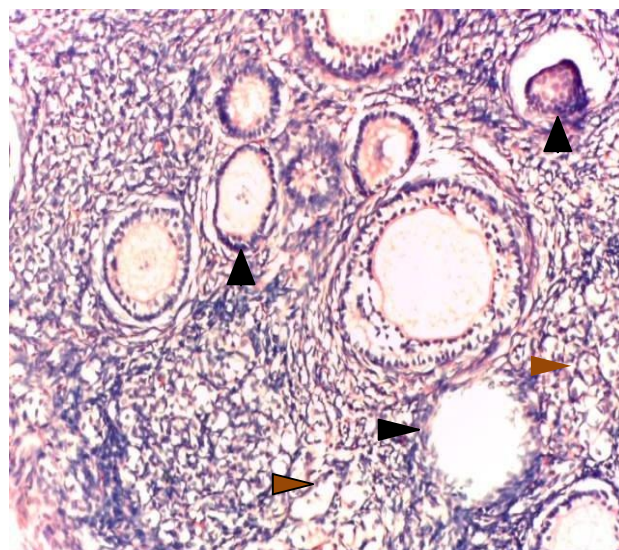
The mean  $\pm$  SD change in body weight per week, ovary weights, and concentrations of serum cholesterol, estradiol and progesterone in the control group were  $0.12 \pm 0.004$ ,  $0.027 \pm 0.003$  kg,  $1.54 \pm 0.15$  mmol/L,  $37.38 \pm 13.29$  pg/ml and  $23.88 \pm 6.88$  ng/l, respectively as shown in Tables 1 and 2. The values of the parameters were significantly affected across the treated groups in a dose dependent manner ( $p < 0.05$ ), especially group C, D and E.

### Histological findings

This can be seen in Figures 1 to 5 and Tables 1 to 3



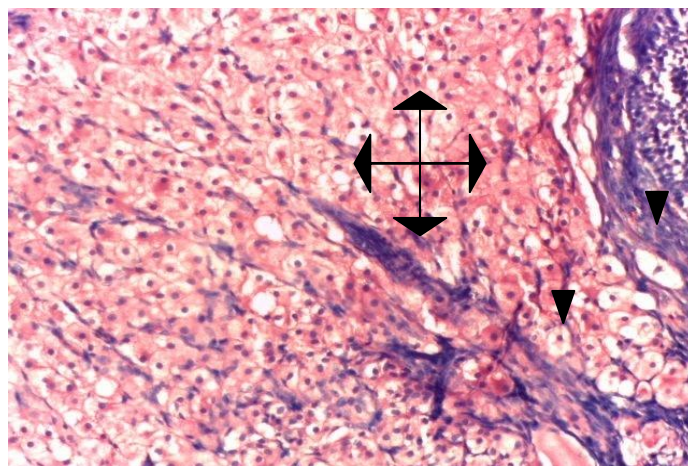
**Figure 3.** Group C (20 mg/kg): Ovarian section with interlacing bundles of closely packed fibrocystic cell types with elongated spindle-shaped basophilic nuclei (arrow). Also observed are hydroptic cells (brown arrow head) in some areas and multi-ovarian cysts (black arrow heads). Stained by H&E Technique.  $\times 200$ .



**Figure 4.** Group D (25 mg/kg): Ovarian section with fibrocystic cell type and cystic spaces. Also observed are hydroptic cells (brown arrow heads). Stained by H&E Technique.  $\times 200$ .

## DISCUSSION

The results of this study highlight the potential susceptibility



**Figure 5.** Group E (30 mg/kg): Ovarian section with multi-hydroptic cells (four black arrows) and fibrosis (black arrow heads). Stained by H&E Technique.  $\times 200$ .

susceptibility of Chinchilla rabbits to reproductive endocrine disruption following exposure to Escravos crude oil, in relation to the measurements of ovary weight, serum concentrations of cholesterol, estradiol and progesterone, and histology of the ovary.

The significant increase in the weight of the ovary observed in this study could be linked to the enlarged follicles (Figure 2) and the hydroptic cells (Figures 3 to 5) seen in the ovary, while the decreased change in body weight (Table 1) in the treated groups could be due to the loss of appetite at the beginning of the study.

Cholesterol is an unsaturated steroid alcohol. The major function of cholesterol is as a metabolic precursor for the biosynthesis of bile acids, and steroid hormones which include male and female sex steroids (androgens and oestrogens) and adrenal steroids (aldosterones and corticosterone) and liver, ovaries, testes and adrenal glands are the main producers of these hormones using cholesterol as the main precursor (Morrissey, 2006). The significant increase in serum cholesterol concentration observed in the treated test group (Table 2) is in concordance with the report of Otiotoju et al. (2011) who administered Bonny light crude oil (BLCO) to female albino Wistar rats. This increase in cholesterol may be an indication of renal retention disease resulting in diminished removal of lipoprotein from the plasma, thus causing the concentration of cholesterol to increase markedly.

Vertebrates synthesize steroids via a pathway that involves the sequential degradation of cholesterol to progestin, then androgens (example testosterone) by the enzyme  $17\beta$  hydroxysteroid dehydrogenase and finally oestrogens (example, 17-oestradiol) by the enzyme

**Table 1.** Mean  $\pm$  SD Body weight of animals (kg) and weight of ovary (kg) in the test and control groups.

Group	Mean change in body weight /week(kg)	P-value	Ovary weight (kg)	P-value
A (control)	0.12 $\pm$ 0.004	-	0.027 $\pm$ 0.003	-
B (15 mg/kg)	0.09 $\pm$ 0.003	0.251	0.028 $\pm$ 0.003	1.000
C(20 mg/kg)	0.02 $\pm$ 0.001	0.002	0.034 $\pm$ 0.003	0.003
D (25 mg/kg)	0.08 $\pm$ 0.004	0.166	0.039 $\pm$ 0.004	0.000
E (30 mg/kg)	0.05 $\pm$ 0.002	0.021	0.034 $\pm$ 0.002	0.004

p-value is significant at ( $p < 0.05$ ). The Mean  $\pm$  SD of the group A (control) was compared with the test groups (across the group) and the level of significance were presented above (p-value). There was a dose dependent increase in the weight of the ovary across the test groups ( $p < 0.05$ ), while there was a decreased Mean change in body weight per week of the test group when compared with the control group.

aromatase (Guillette et al., 1994). Progesterone is produced in the ovaries (by the corpus luteum), the adrenal glands and during pregnancy (by the placenta). It plays a vital role in the maintenance of pregnancy in part because it suppresses uterine contractility and most importantly because it contributes to a state of "transplantation immunity" and prevents immunological rejection of the fetus (Siiteri and Macdonald, 1973). The reports by Alvarez et al. (2000) stated that crude oil acts as an anti-androgenic compound, and thereby inducing spontaneous abortion, stillbirths and reproductive malfunction, following the ingestion of hexachlorobenzene (a component of crude) by female rats.

These reproductive malformation and developmental disorders are the results of adverse effects on endocrine system (Indarto and Izawa, 2001). Previous study has shown that ingestion of crude oil contaminated water and feed by rats produced endometrial changes; thick endometrial epithelium and cornification as well as keratinized papilloma resulted in foetal abortion (Oveh and Nwankwoala, 2009). In this study, the significant decrease in the level of progesterone (Table 2) observed in the female test groups is in concordance with the findings of Georgewill and Nwankwoala (2006) who worked on BLCO. The decrease in progesterone may be due to formation of cystic spaces in the ovary; a pathologic replacement of functional hormone producing cells and the formation of interlacing bundles of closely packed fibrocystic type cells as shown in the photomicrographs (Figures 2 to 4).

Animals orally given the Nigerian Qua-iboe Brent crude oil exhibited irregular oestrous cycle when assessed via vaginal cytology, with subsequent significant effects on the conception rates (decrease), gestation length (increase) and litter size (decrease) in the test group when compared with the control group (Nwaigwe et al., 2012).

In this study, the test animals had significantly high

concentration of estradiol (Table 2). This is not in accordance with the results of Georgewill and Nwankwoala (2006), who reported a decrease in the level of oestrogen in female guinea pigs which were given 3 kg feed/500 ml Nigerian Agip oil. The increase in estradiol is related to the increased serum cholesterol level, as there is a strong correlation between both variables. It could be adduced that the cholesterol glut made the production of more estradiol feasible. The concentration of progesterone was found to be inversely proportional to the concentration of cholesterol, a negative correlation (Table 3).

This could mean that the concentration of the Escravos crude oil causes a dose dependent inhibition of the enzyme responsible for the production of progesterone. According to the findings of Wagner et al. (1994), alopecic ferrets had high serum estradiol concentration. Some of the animals in the treated groups D and E showed signs of alopecia (hair loss) which could be associated with the high serum estradiol concentration observed.

A drop in progesterone level could be one step that facilitates the onset of labor (Luoma et al., 2012; Herson et al., 2009). Asara et al. (2013) reported that the loss of pregnancy following ingestion of BLCO contaminated feed might be explained by the crude oil induced destruction of the lining of epithelial from the fallopian tubes and on the ovarian stroma that was seen in the ovaries. Progesterone modulates the activity of CatSper (cation channels of sperm) voltage-gated  $Ca^{2+}$  channels. Since eggs release progesterone, sperm may use progesterone as a homing signal to swim toward eggs (chemotaxis). Hence substances that block the progesterone binding site on CatSper channels or decrease its concentration in the blood could potentially cause infertility in female (Strünker et al., 2011).

The histological assessment of the sectioned ovaries in this study shows multiple cysts (immature follicles) of various sizes and features of necrosis (ballooning degeneration/hydropic cells) and fibrosis (Figure 5).

**Table 2.** Mean  $\pm$  SD serum and pairwise comparisons of the concentrations of sex hormones and cholesterol.

Parameter	Group A (control)	Group B (15 mg/kg)	Group C (20 mg/kg)	Group D (25 mg/kg)	Group E (30 mg/kg)	f-value	p-value
Cholesterol (mmol/l)	1.54 $\pm$ 0.15	2.18 $\pm$ 0.47 (0.007)	2.31 $\pm$ 0.27 (0.002)	2.33 $\pm$ 0.28 (0.001)	2.96 $\pm$ 0.17 (0.000)	12.166	0.000
Estradiol (pg/ml)	37.38 $\pm$ 13.29	38.68 $\pm$ 6.45 (0.875)	41.42 $\pm$ 15.05 (0.626)	45.86 $\pm$ 8.44 (0.312)	68.86 $\pm$ 11.96 (0.001)	5.089	0.009
Progesterone (ng/l)	23.88 $\pm$ 6.88	21.57 $\pm$ 3.41 (0.454)	15.45 $\pm$ 0.17 (0.000)	9.80 $\pm$ 3.29 (0.000)	10.45 $\pm$ 4.50 (0.000)	20.293	0.000

P -value is significant at ( $p < 0.05$ ). The pairwise comparison was made between the control group and the treated groups. The post hoc test (LSD) showed significant decrease in serum progesterone concentration with significant increase in serum cholesterol and estradiol concentrations ( $p < 0.05$ ), in a dose dependent manner across the treated group.

**Table 3.** Correlation between cholesterol, estradiol and progesterone.

Variable	r-value	p-value	Remark
Cholesterol correlated with Estradiol in females	0.539*	0.014	Positive correlation
Cholesterol correlated with Progesterone in females	-0.381	0.098	Negative correlation

\*Correlation is significant at the 0.01 level (2-tailed). Pearson's correlation. The correlation between the concentrations of sex hormones and cholesterol showed that the increase in cholesterol concentration led to a proportional increase in the concentration of estradiol but led to an inverse decrease in the serum progesterone concentration.

## Conclusion

Hormones act at extremely low levels (part per trillion), therefore exposure to low levels of hormonally active agents as found in the crude oil may be of major health concern, particularly during sensitive periods of development and reproduction. The elevated cholesterol, estradiol levels and ovary cysts found in this study are suggestive of polycystic ovarian syndrome. The increased weight of the ovary might be related to the increased number of follicles found in the ovaries. This study suggests that Escravos crude oil, a variant of the Nigerian crude oil, might possess estrogenic property and may be a potential toxic substance to the female reproductive organs.

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