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Effects of Crude Oil contaminated Water on the reproductive system of Female Wistar Rats

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ABSTRACT

The rising level of infertility in the females have been associated with environmental pollutants and one such pollutants is crude oil. Crude oil pollution occurs in the Niger-Delta region of Nigeria and interaction with this toxicant by both humans and animals may adversely the female reproductive system. This study was designed to investigate the effect of crude oil contaminated water on reproductive functions in female Wistar rats. Fifteen (15) female rats weighing between 140g-230g were used for this study and the rats were divided into three (3) groups (1, 2 and 3) and each group had five rats. Group 1 was the control group while group 2 and 3 were the test groups receiving 2.5mls and 5mls of crude oil contaminated water twice daily for four weeks. The stages of Oestrus cycle of the rats were checked daily. The rats were sacrificed after four weeks and the blood collected were used for hormonal assays (Follicle stimulating hormone (FSH), Luteinizing hormone (LH), Testosterone). The uterus and ovaries were used for histology (Haematoxylin and Eosin staining), Superoxide dismutase (SOD) activity and Malondialdehyde (MDA) concentration. The results were analyzed using GraphPad Prism software for windows and data were expressed as mean±standard error of mean. Statistical significance was accepted at $p<0.05$.

There was no significant changes ($p>0.05$) in the weights and relative reproductive organ weights (uterus and ovary) of among the three groups. FSH and LH in the test group of rats fed with crude oil contaminated water when compared with the control were not statistically significant ($p<0.05$). Oestradiol was significantly lower ($p<0.05$) in the test group whereas progesterone and progesterone-oestradiol ratio was significantly elevated ($p<0.05$) in the test groups when compared with the control. There were degenerative changes in the ovaries and uterus of rats fed with crude oil contaminated water when compared with the control. SOD was significantly reduced ($p<0.05$) in the test group while MDA was elevated in the ovaries and uterus of the test group. The oestrus cycle was irregular in the test groups.

Crude oil contaminated water has endocrine disrupting effects on the female reproductive system as well as inducing oxidative stress and this may adversely affect female reproductive functions and fertility.

KEY WORDS: Crude oil contaminated water, Endocrine disruptor, Female reproductive functions, Oxidative stress

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Introduction

Reproduction is fundamental for the life of an individual as well as the survival and development of the species (Roychoudhury and Massányi, 2014). The reproductive system controls the morphological development and physiological differences between males and females as well as influences the behavior of the organism. The ovary is responsible for regulating reproduction through the coordinated development and release of a mature oocyte (folliculogenesis) and is responsible for regulating menstrual/estrus cyclicity and sexual behaviour/characteristics through the synthesis and secretion of steroid hormones (steroidogenesis) (Gibson and Mahdy, 2021). These processes are under control from the hypothalamic pituitary ovarian (HPO) axis, whereby gonadotropin releasing hormone from the hypothalamus facilitates the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary to aid in follicular growth and ovulation (Abedel Majed *et al.,* 2019; Richards and Pangas, 2010). The hormones produced by the hypothalamic pituitary ovarian (HPO) axis can be influenced by environmental contaminants known as endocrine disruptors. An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) population (WHO, 2002). The homeostasis of sex steroids and the thyroid are the main targets of EDC effects; hence, reproductive health, considered as a

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continuum from gamete production and fertilization through to intrauterine and post-natal development of progeny, is recognized as being especially vulnerable to endocrine disruption (Mantovani, 2002). EDCs are widespread in food chains and in the environment and include persistent organic pollutants (POPs) such as the insecticide dichlorodiphenyl-trichloroethane (DDT) and its metabolites, the industrial by-product dioxins and the industrial compounds polychlorinated biphenyls (PCB), several agrochemicals, pesticides and biocides (e.g. chlorinated insecticides, organotins, imidazoles and triazoles) and other industrial compounds (several phenol compounds such as bisphenol A) (Mantovani et al., 1999). Crude oil is an environmental toxicant with huge public health concerns which includes reproductive toxicity. Crude oil is a complex mixture consisting of several hydrocarbons and non-hydrocarbon materials that may be refined into several types of petroleum products that eventually becomes sources of energy and raw materials for chemical manufacturing industries (Overton *et al.,* 2016). Several heavy metals have been reported in crude oils. Some of these heavy metals include zinc, vanadium, lead, manganese, cobalt, iron, nickel, and cadmium (Udeme and Udoessien, 2012). Crude oil is very important in various parts of the world and its products are used as fuel for automobiles. It is also useful in the generation of electric power (Ohanmu *et al.,* 2019). In Nigeria, crude oil is produced in the Niger-Delta region and this region has a land mass of about 70,000km^3, 7.5% of the total land mass of Nigeria with two rivers (rivers Niger and Benue), that drain into the Atlantic Ocean. The Niger-Delta has a population of about 25 million people occupying 9 states and 186 local government areas (Ayanlade, 2012; Enegide *et al.,* 2018).

Despite the benefits of crude oil and its products, oil pollution resulting from crude oil spillage is a major problem encountered on a regular basis by the crude oil producing communities of the Niger-Delta. These occur during the process of crude oil exploration and exploitation. The adverse effects of crude oil extend to both aquatic and terrestrial organisms (Ogeleka *et al.,* 2017). Over the past 50 years, an estimated 9-13 million barrels of crude oil has been spilled in the Niger-Delta and this spill occur on land, swamp and offshore environment (Ogeleka *et al.,* 2017).

This study is designed to evaluate the effects of crude oil contaminated water on female reproductive system and functions.

Materials and Methods

Animals

Fifteen (15) female rats weighing between 140g-230g were used for this study. The rats were kept in Lagos State University College of Medicine (LASUCOM) Animal House and maintained at room temperature, adequate

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humidity and with 12 hours dark and 12 hours light cycle. The rats were fed standard rat chow and water *ad labitum*. The study was approved by LASUCOM Animal Research Ethics Committee.

Experimental design

The rats were divided into three groups (1, 2 and 3) with each group having five (5) rats each. Group A was the control while groups B and C were the test groups. Group A received distilled water, while groups B and C were given daily 2.5mls and 5mls of crude oil contaminated water respectively via oral gavage twice (day and night) for a total of 5mls and 10mls for four weeks. Crude oil contaminated water was obtained from Abonema river in Rivers State, Nigeria.

After four weeks, all groups were sacrificed. The rats were administered intraperitoneally with 0.2ml/100g of Ketamine HCl. (de Carvalho et al., 2011) and were monitored for signs of surgical anaesthesia before sacrifice. 3.5 Blood samples of each rat were collected through cardiac puncture. The sera were separated from the blood cells, aliquoted into Eppendorf tubes and kept at -20°C until used in hormonal assay. The ovaries and uteri were dissected free of fat and weighed using a electronic weighing balance. Some tissues obtained were preserved in 10% buffered formalin for histology using Heamatoxylin and Eosin staining (H&E) while the other samples of the uterus and ovaries were separately stored in -20°C until they were homogenized for antioxidant enzymes assays.

Hormonal Assay

The Follicle Stimulating Hormone (FSH), Luteinizing hormone (LH), Oestradiol and progesterone assay were carried out using the sera from the groups. The procedure had previously been described (Ajonuma *et al*. 2017a, Ajonuma *et al* 2017b). Briefly, hormonal assay enzyme linked immunosorbent assay (ELISA) kits used were purchased from Monobind Inc., CA, USA. The assays were done according to the instructions of the manufacturer. Assay kits were brought to room temperature. The following steps were taken: The desired numbers of micro strips were put into frame; wells were allocated for each serum sample and 12 wells for the calibrator samples. 50µl of Calibrator sample were placed into the wells using pipette. 50µl of serum were also placed in the required wells. 50µl of hormonal Conjugate Reagent was dispensed into the wells and then incubated for 60 minutes at 37 degree Celsius. After incubation, the strips were washed five times with prediluted washing solution (containing surfactant in buffered saline). The washing solution was prepared by 20x dilution of washing solution concentrate using distilled water. 50µl of 3,3,5,5'-Tetramethylbenzidine (TMB)

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substrate A solution was added into each of the wells followed by the addition 50µl of TMB Substrate B solution into all the wells. This was well mixed and incubated at 26 degree Celsius for 20 minutes. 100µl of stop solution was added into each of the wells using a pipette. Optical density (OD) was measured at 450nm/620nm on a STAT Fax 4700 ELISA micro plate reader.

Superoxide dismutase (SOD) Assay

The assay of SOD was carried out as described by Maklund and Maklund, (1974). It is based on the antioxidation of pyrogallol in a basic medium. The Spectrophotometer was adjusted to zero reading using Tris-EDTA buffer, the control (distilled water) and the homogenate were added into the test tubes. 25µL of control sample and homogenate was added separately to 500µL of TrisEDTA buffer (PH= 8.2) and this was followed by the addition of 0.2mM of pyrogallol. Absorbent was read at wavelength of 420nm against Tris-EDTA buffer at zero time and after one minutes of addition of pyrogallol.SOD activity was calculated as percentage inhibition of pyrogallol autoxidation by the homogenate.

Malondialdehyde (MDA)

Malondialdehyde concentration was determined in the homogenate of the ovary and uterus using the method previously described by Buege and Augst (1978). 0.37% of Thiobarbituric (TBA) acid, 0.24N hydrochloric acid (HCI) and 15% tricarboxylic acid (TCA) were mixed in the ratio 1:1:1 (TBA (0.37%): 0.24N HCI: 15% TCA). This mixture was added to the homogenate in the ratio 1:2 (homogenate 1: mixture 2). The new mixture was boiled at 100°C for 15 minutes and then allowed to cool. This was then centrifuged at 3000rpm for 10 minutes. The supernatant was decanted using a pipette, and the absorbance of the supernatant was read at the wavelength of 532nM against a blank. The MDA was calculated using the extinction coefficient for MDA TBA complex of 1.56 X 10- M-1 cm-1.

Heamatoxylin and Eosin (H & E) tissue staining

This was carried out on the ovary and uteri that were previously fixed in 10% formalin as described Ajonuma et al. (2017a, 2017b) with modifications. Fixed tissue is transferred to the mould containing paraffin wax and the wax was blown on the surface until a thin film of wax solidified. The mould containing the tissue was transferred to a container of cold water. It remained submerged until the wax hardens. The paraffin block was trimmed and placed on ice for 1 hour and the block was fixed in position on the microtome and sections were cut at 5µm. The

thin section is floated with 20% alcohol in a warm bath at 40°C. The thin section was picked and dried on hot plate at 75°C. The section was taken into water and stained in Haematoxylin for 10 minutes. The stained section was rinsed in water and differentiated in 1% acid – alcohol. This was rinsed in water for 1 minute and counter stained with 1% Eosin for 1 minute. The Excess stain was washed, and the section was dehydrated in ascending order with 70%, 90%, 100% alcohol for 15 seconds each, the section was cleared in Xylene and mount in dihydroxy phthalate xylol (DPX). The stained section was evaluated using a light microscope (Olympus) and images of the sections were captured using attached camera.

Oestrus cycle Determination

The vaginal swear was obtained by using cotton-tipped swab wetted with normal saline and introduced into the vagina of the restrained rat. The swab was gently turned and rolled against the vagina wall and then removed. Cells collected were then transferred to a dry glass slide by moving the swab across the slide. The slide was air-dried, fixed in methanol stained using both field stain A and B viewed under the microscope (Ajonuma *et al.* 2018). The phases of the oestrus cycle were then appropriately determined.

Statistical Analysis

Results were presented as mean SEM. The mean across groups were analysed using Analysis of Variance (ANOVA) and Turkey post hoc test. Statistical significance was accepted at $p \leq 0.05$. Statistical analyses were carried out using Graph Pad Prisms, version 8, Graph Pad Inc, USA.

Results

Effect of crude oil contaminated water on percentage weight change, Relative uterus and ovarian weights of Female rats.

There was no significant difference ($p > 0.05$) in the percentage weight change across groups 1 to 3. However, a pattern of reduction was observed in the test groups 2 and 3 when compared to the control group (Fig 1.a).
There was no significant change ($p>0.05$) in the relative uterus weight of rats in the test group when compared to the control group. However, a pattern of reduction in the test groups 2 and 3 was observed (Fig 1.b).
There was no significant change ($p>0.05$) in the relative ovary weight of rats in the test group when compared to the control group. However, a pattern of reduction in the test groups 2 and 3 was observed (1.c)

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The Effects of crude oil contaminated water on Serum Progesterone, Estradiol and Progesterone / Estradiol ratio, FSH and LH.

Crude oil contaminated water caused a significant elevation ($p<0.05$) of serum progesterone level in the test groups 2 and 3 when compared to the control group 1 of female rats (Fig. 2a).

There was a significant decrease ($p<0.05$) in the serum estradiol level in female rats of test groups 2 and 3 when compared to the control group (Fig. 2b).

There was a significant increase ($p<0.05$) in progesterone-estradiol ratio in group 2 and 3 when compared to the control group (Fig. 2c). .

Crude oil contaminated water caused a non-significant elevation ($p>0.05$) in the serum level of FSH across rats in test groups 2 and 3 when compared with the control group (Fig. 3a).

Crude oil contaminated water caused a non-significant elevation ($p>0.05$) in the serum level of LH across rats in test groups 2 and 3 when compared with the control group (Fig. 3b).

Effects of effects crude oil contaminated water on the Histology of the uterus There were normal glands in the uterus of group1 (control).

Crude oil contaminated water caused multiple cystic glands in the uterus of group 2 (2.5mls) (fig. 4). Crude oil contaminated water caused multiple cystic glands in the uterus of group 3 (5mls) (fig. 4).

Effects of effects crude oil contaminated water on the Histology of the Ovary There was developing follicles with normal oocytes in the ovary of the group1 (control group) (fig. 5). Crude oil contaminated water caused degenerating or atretic follicle developing follicles with degenerating or atretic oocytes in the ovary of group 2 (2.5ml) (fig. 5). Crude oil contaminated water caused degenerating follicle, cystic with degenerating or atretic oocytes in the ovary of group 3 (5ml) (fig. 5).

Effects of effects crude oil contaminated water on Uteri and Ovarian Superoxide Dismutase and Malondialdehyde Concentrations of Female Wistar rats

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Crude oil contaminated water caused a significant elevation ($p<0.05$) in malondialdehyde concentration and suppression of superoxide activities in uterus of female Wistar rats in groups 2 and 3 when compared to the control.

Crude oil contaminated water caused a significant elevation ($p<0.05$) in malondialdehyde concentration and suppression of superoxide activities in ovary of female wistar rats in groups 2 and 3 when compared to the control

Effects of effects crude oil contaminated water on Oestrus cycle of female Wistar rats

Estrus cycle for most of the rats in control group ranges from four to six days, but there is prolonged estrus cycle in test group (groups 2 and 3) exceeding six days with persistent oestrus phase being the predominant phase of the oestrus cycle.

Discussion

This study evaluated the effects of chronic oral exposure to crude oil contaminated water on female reproductive system of Wistar rats. There was no significant change in the weight and relative reproductive organs weights (ovary and uterus) in both control and test groups. However there was increase in weight gain in controls when compared with test groups and this is similar to the results of Raji and Hart (2012).

There were no significant changes in both luteinizing hormone and follicle stimulating hormone, but they appear to be higher in the test groups. This probably suggests that crude oil may not be affecting gonadotropins directly.

Although there was no change in relative weight of both ovaries and uterus, there were degenerative changes in both organs. The degenerative changes in ovary of crude oil contaminated water include; Atretic changes in the follicle with associated degenerating oocyte that agrees with findings of Okoye et al (2014). Several components of crude oil contaminated water in the form of polycyclic aromatic hydrocarbons (PAH) and heavy metals cause degenerative changes. Exposure to benzopyrene (BaP), a PAH found in crude oil caused degenerative changes in the ovaries of mice and the changes include pyknosis and cytolysis of the oocytes in

primodial follicle stage (Mattison et al 1980). BaP exposure has also been shown to impair meiotic progression in both porcine (Miao et al., 2018) and murine oocytes, compromising oocyte maturation and quality through altered spindle assembly, chromosomal alignment, cytoskeleton structures and mitochondrial integrity (Sui et al., 2020; Sobinoff et al., 2012; Zhang et al., 2018). Heavy metals present may also have contributed to these findings. Cadmium exposure to female rats and rabbits have been shown to cause decrease in volume of growing follicle, damaged and fewer oocytes and increased number of atretic follicle (Nna et al. 2017; Nasiadek et al. 2019; Massanyi et al. 2020; Ruslee 2020). Lead and Mercury exposure have also been documented to produce similar ovarian changes in exposed female rodents (Uchewa and Ezugworie, 2019; Merlo et al. 2019). The synergistic effect of these components may account for these changes seen in the ovaries. These changes in the ovaries may alter hormones secreted in the ovary (oestrogen and progesterone) and the process driven by these hormones may be affected by any alteration in their secretion. Interestingly, ingestion of crude oil contaminated water may lead to meiotic arrest during folliculogenesis.

Uterine changes include degenerative and cystic formation in uterine glands. These glands secrete nutrients in preparation for implantation of embryos (Sembulingam, 2012). When these glands are degenerated, it means the capacity of uterus to receive and adequately accommodate the embryo will be impaired.

There was significant elevation of estradiol in control when compared with test groups, this finding agrees with those of Nyeche et al. (2015) but disagrees with the findings of Okoye et al. (2014). This may be due to the effects of crude oil contaminated water on the ovary. BaP a component of crude oil causes a reduction in the production of estradiol (Archibong *et al.* 2012). Although we did not analyze the contents of the present batch of crude oil contaminated water, it may be that the levels of BaP and other components that cause decreased estradiol levels may have been high. However, this is not common.

The reduction in estradiol implies that all the functions performed in the female by this hormone will be suppressed. Some of the functions include enlargement of the uterus so that growing foetus can be accomomodated (Sembuligam and Sembuligam, 2012).

Progesterone was progressively elevated in test groups when compared with the control. This finding agrees with the findings of Nyeche et al, (2015) but disagrees with findings of Okoye et al. (2014). Progesterone performs many reproductive functions; however, elevated progesterone level causes infertility by increasing

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the thickness of mucosa in the cervix, which is not favorable for transport of sperm (Sembuligan and Sembuligan, 2012) and this may contribute to subfertility in rats exposed to crude oil.

Progesterone-estradiol ratio is elevated in test groups. To the best of our knowledge, this is the first study to compare the ratio in crude oil contaminated water study. The ratio is important because elevated progesterone-estradiol ratio has been associated with reduced clinical pregnancy and poor fetal outcomes (Keltz et al. 2012). This implies that in rats that were administered crude oil, fertility may be impaired as a result of elevated progesterone-estradiol ratio.

Several studies in our laboratory (unpublished data) showed that the effects of crude oil and crude oil contaminated water are mediated via oxidative stress. In this study, there was elevated MDA in the uterus and ovaries of the test group when compared to the control and the reduced SOD in uterus and ovaries of the test groups when compared with the control. This redox imbalance implies that there is oxidative stress in both ovaries and uteri of rats exposed to crude oil contaminated water. Cadmium may contribute to the oxidatives stress seen in these organs as it has been reported to cause decrease in antioxidant enzymes and increase MDA (Nna *et al.* 2017). The consequences of oxidative stress in female reproductive system are very many and they include poor oocyte quality, poor corpus luteum, infertility, unexplained infertility, poor embryo development, abortion, affect progression of cell division in fertilized oocytes (Agarwal *et al.,* 2012). Women exposed to crude oil contaminated water may become infertile as a result of elevated oxidative stress.

Oestrus cycle was irregular and prolonged for most of the rats in the test groups and this finding agrees with the study (Raji and Hart 2012, Nyeche et al 2015, Okoye et al., 2014). The possible cause of the irregular oestrus cycle is similar to the endocrine disruptor effects of crude oil as seen in the abnormal hormone production of the rats. The hormone drives the oestrus cycle and as a result, the oestrus cycle will be altered following hormonal changes.

The study demonstrated that crude oil contaminated water has a negative effect on reproductive functions of female Wistar rats. It is not known whether these reproductive impairments caused are reversible. Further studies are needed to elucidate the present findings.

In summary, this study has demonstrated that ingestion of crude oil contaminated water had adverse effects on reproductive organs of the female Wistar rats, and interfered with secretion of reproductive hormones, thus leading to impaired or irregular oestrus cycles. The degree of toxicity may be related to both frequency

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and dose of administration. These findings may lead to female infertility in those drinking from crude oil contaminated water sources.

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Figures and Tables

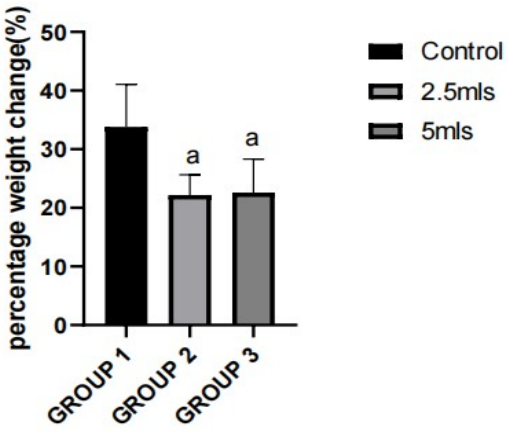

Fig 1.a. The effect of Crude oil contaminated water on percentage weight change of female rats. [a]p<0.05, n=

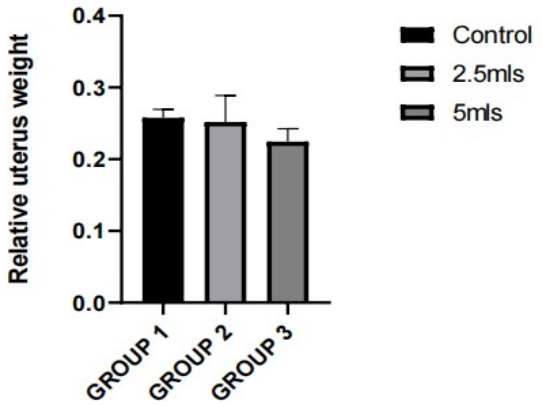

Fig 1.b. The effect of Crude oil contaminated water on relative uterine weight of female rats. P>0.05, n=5

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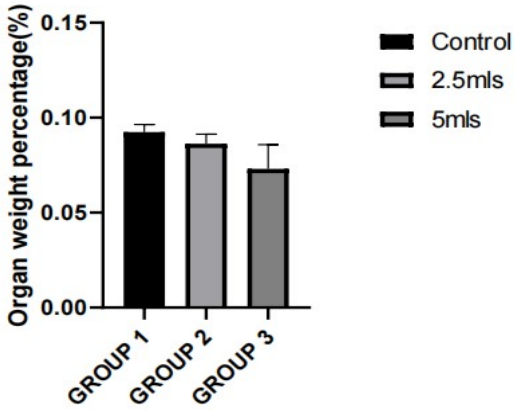

Fig 1.c. The effect of Crude oil contaminated water on relative ovary weight of female rats. P>0.05, n=5

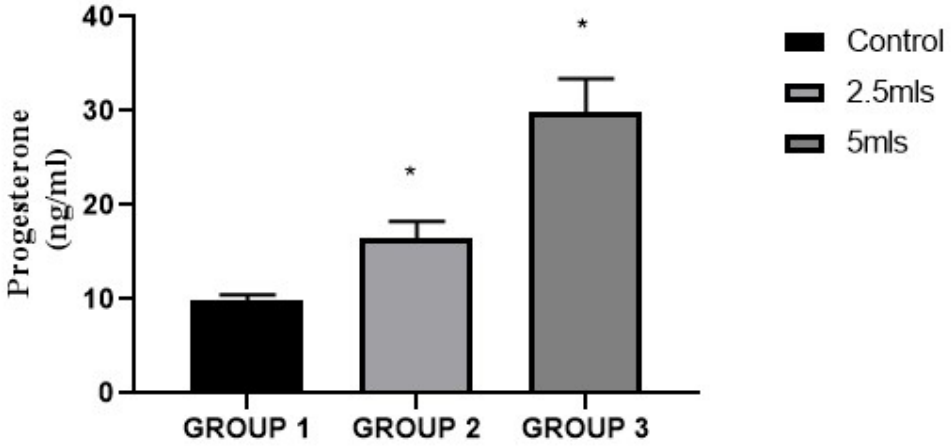

Fig 2.a. The effect of Crude oil contaminated water on serum Progesterone of female rats. *p<0.05, n=5

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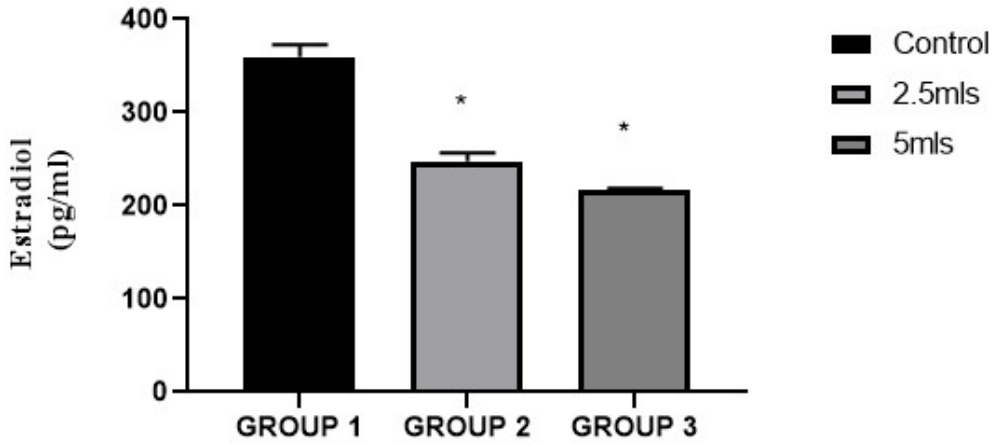

Fig 2.b. The effect of Crude oil contaminated water on serum Estradiol of female rats. *p<0.05, n=5

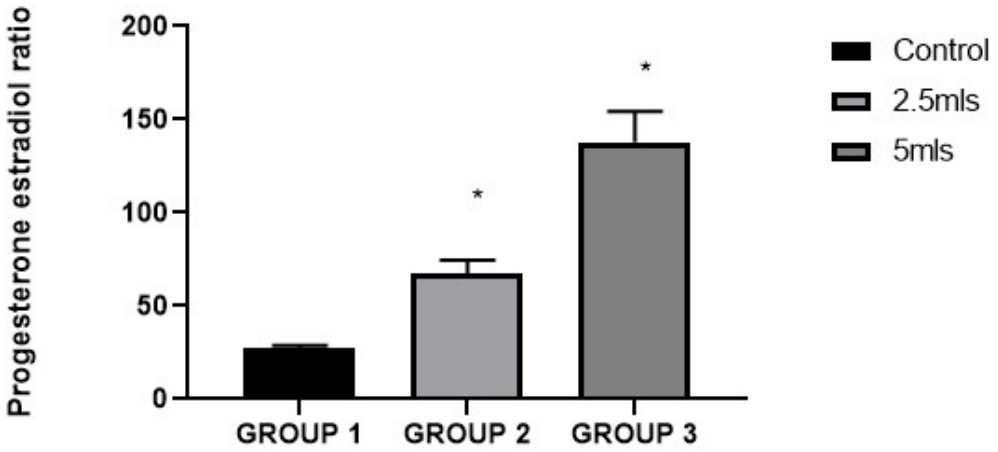

Fig 2.c. The effect of Crude oil contaminated water on serum Progesterone estradiol ratio of female rats. *p<0.05, n=5.

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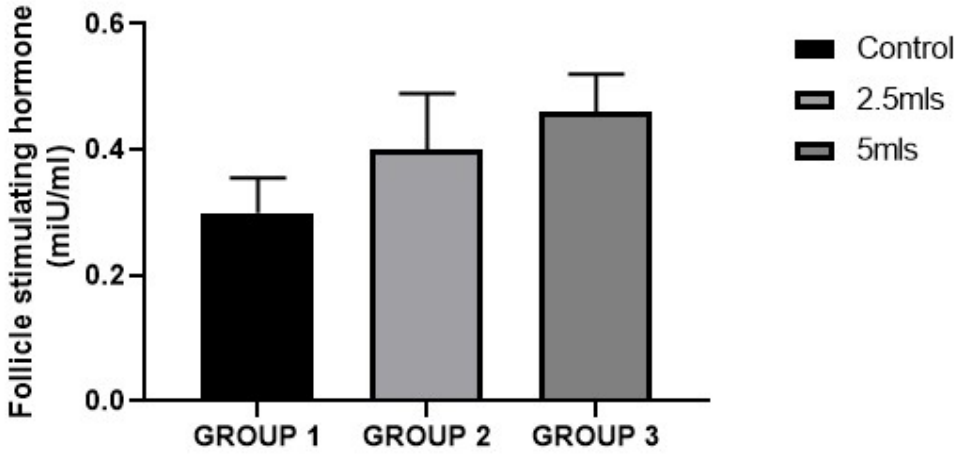

Fig 3.a. The effect of Crude oil contaminated water on serum FSH of female rats. P>0.05, n=5

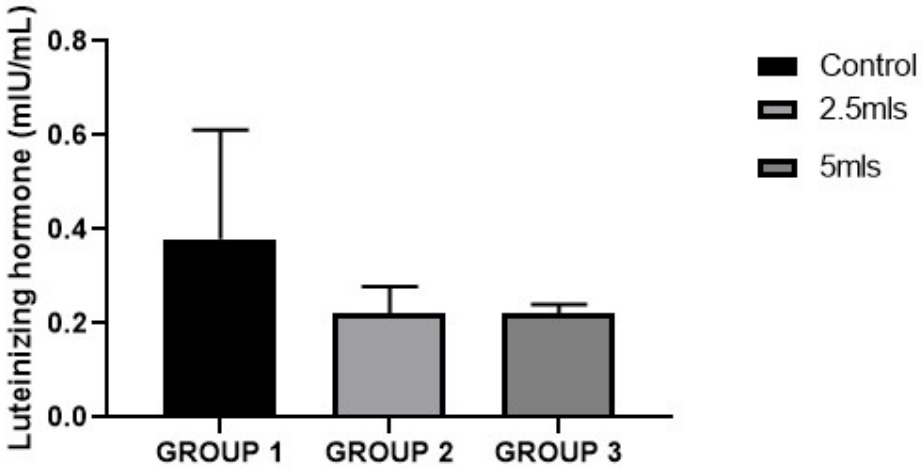

Fig 3.b. The effect of Crude oil contaminated water on serum Luteinizing hormone of female rats. P>0.05, n=5

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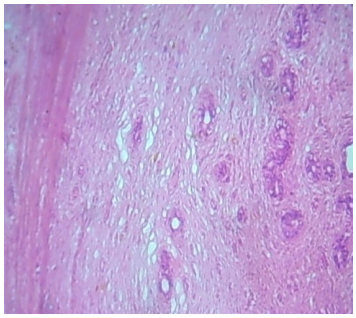

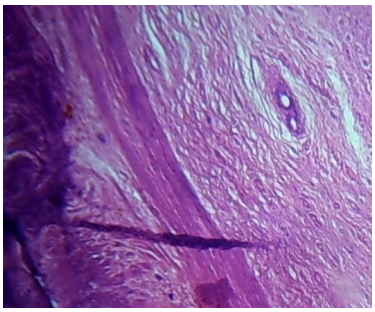

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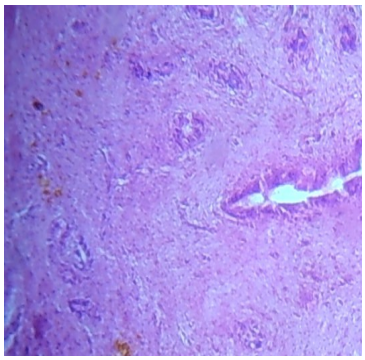

Fig 4. Photomicrograph of H&E stained section of uteri of group 1 (control), Crude oil contaminated water caused multiple cystic glands in the uterus of group 2 and multiple cystic glands in the uterus of group 3. Magnification 10X.

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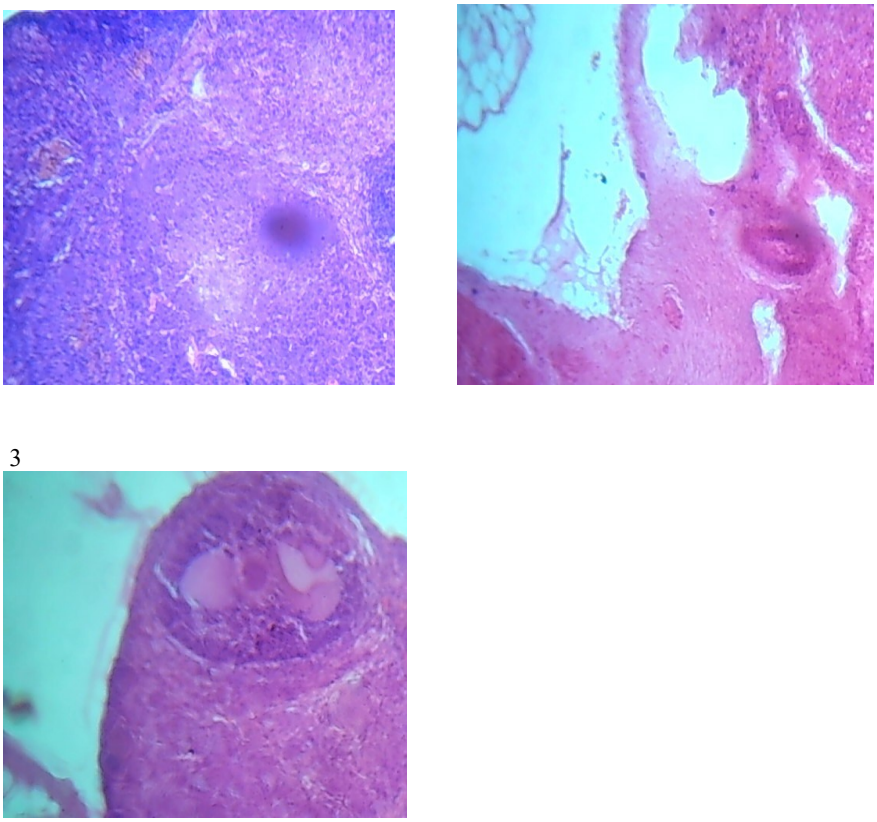

Fig. 5. Photomicrograph of H&E stained section of ovaries of group 1 (control), group 2 and 3 (test groups). There was developing follicles with normal oocytes in the ovary of the group1 (control group).Crude oil contaminated water caused degenerating or atretic follicle developing follicles with degenerating or atretic oocytes in the ovary of group 2 and 3. Magnification 10X.

Table 1. Effect of crude oil contaminated water on uterine antioxidant (Superoxide dismutase) and malondialdehyde concentration

Uterine Parameters	GROUP 1	GROUP 2	GROUP 3
Superoxide dismutase (µmg/protein)	0.83±0.02	0.70±0.08*	0.10±0.09*
Malondialdehyde concentration (µmol/g)	1.31±0.10	6.08±0.37*	9.43±0.63*

*p<0.05, n=5

Table 2. Effect of crude oil contaminated water on Ovary antioxidant (Superoxide dismutase) and malondialdehyde concentration

Ovary Parameters	GROUP 1	GROUP 2	GROUP 3
Superoxide dismutase (µmg/protein)	1.87±0.01	1.80±0.01*	1.71±0.01 *
Malondialdehyde concentration (µmol/g)	9.00±0.47	14.69±0.35*	22.08±0.84*

*p<0.05, n=5