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Full Length Research Article

Maternal exposure to Bonny Light Crude Oil Altered Reproductive indices in Male and Female offspring of Wistar rats

***Jeje S.O.[1], Ukwenya V.O.[2], Agbaje A.B.[1] and Daramola O.M.[1]**

Departments of [1]Physiology and [2]Human Anatomy, School of Health and Health Technology, Federal University of Technology, Akure, Nigeria

Summary: In this study, the effects of maternal exposure to Bonny Light Crude Oil (BLCO) on reproductive functions of the offspring was investigated in Wistar rats. Ten pregnant rats were divided into two groups (n=5). Group 1 served as the control, it was administered 0.75ml/Kg bwt/day normal saline and Group 2 was administered 0.75ml/Kg bwt/day BLCO. Serum hormonal profile, sperm indices, estrous cycle length and pubertal timing were assessed as measures of reproductive function. Tissue Malondialdehyde, Catalase and SOD activities were assessed as indices of oxidative stress. Results obtained showed that BLCO significantly ($p<0.05$) reduced birth weight, anogenital distance (AGD) at birth, sperm count, motility and normal morphology, serum testosterone, testicular and epididymal SOD and catalase activities in the male offsprings. However, days of preputial separation, relative weight of testis and epididymis, testicular and epididymal MDA were significantly ($p<0.05$) raised by gestational exposure to BLCO. In the female offspring, birth weight, AGD at birth, relative weight of ovaries and uterus, SOD, catalase activities, serum LH were significantly reduced by BLCO exposure during gestation. Moreover, uterine and testicular MDA, serum estradiol and FSH were significantly increased by BLCO treatment during gestation. In conclusion, maternal exposure to BLCO during gestation may alter reproductive indices in the offspring and increased occurrence of oxidative stress in reproductive structures in male and female offspring of Wistar rats.

Keywords*: Bonny Light Crude Oil, Reproductive function, offspring, gestation, oxidative stress*

**Address for correspondence: sojeje@futa.edu.ng; Tel: +2348086327115*

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INTRODUCTION

Infertility is a clinical problem that affect people medically, and also affects the family stability. According to Giwa-Osagie (2003) about 10-25% of couples in African countries are sub-fertile and male factor infertility accounts for 30-40% of these cases. Recent studies have shown that reproductive dysfunction can be epigenetically induced due to exposure to environmental agent such as heavy metals and other environmental pollutants including crude oil (Ola-Mudathir *et al*., 2008; Fischer *et al*., 2013). Exposure of humans to disruptive chemicals appears to be related to various reproductive health problems such as decreased fertility, menstrual disorders, impaired spermatogenesis, cryptorchidism, hypospadias, low birth weight, structural and functional birth defects and postnatal developmental defects (Kumar, 2008). Research from a wide range of scientific disciplines have shown that the reproductive performance of animals at adult life is determined, in part, by a variety of endocrine disruptors acting at different stages of development from fetal to early neonatal life (Jeje and Raji, 2017). These effects are probably mediated through changes in the hypothalamic-pituitary-gonadal axis but the physiological system that is affected depends on the stage of development at which the influence is applied (Stewart *et al*., 2001).

Bonny Light Crude Oil (BLCO) is characterized by low Sulphur content and low corrosive property (Fischer *et al*., 2013). It was first associated with Bonny Island area of southern edge of Rivers State in the Niger Delta region of Nigeria. In Nigeria, frequent oil spills resulting from pipeline vandalism, theft and poor maintenance are major source of environmental pollution. When crude oil or other petroleum products leak into the environment, the different compounds evaporate into the air and are absorbed by the soil and water, crop or fish consumed by humans. Previous studies have suggested that BLCO is a potent reproductive toxicant and an anti-androgenic agent (Orisakwe *et al*., 2004; Fischer *et al*., 2013). In addition, BLCO induced alterations in liver mitochondria DNA concentration and increased the binding of nickel to chromatin proteins in guinea pig (Oruambo *et al*., 2007). Therefore, crude oil and its constituent hydrocarbons have been suggested to be responsible for at least some component of infertility/ sub - fertility in the Nigerian population. This study is aimed at investigating the effect of maternal exposure to BLCO during gestation on reproductive function of the offspring in Wistar rats.

MATERIALS AND METHODS

Animals: Adult male and female Wistar rats (10 weeks old, weight 180-200g and 150-170g for male and female respectively) obtained from the Department of Physiology Animal House, Federal University of Technology, Akure, Nigeria were used. These animals were housed in cages in the Department of Physiology Animal House and had access to food (Ladokun Feeds Limited, Ibadan, Nigeria) and water for the entire duration of the study *ad libitum*. The females were nulliparous and the males used for the mating were

proven male breeder. The animals were kept under standard laboratory condition. Animals were allowed to acclimatize for 2 weeks to the laboratory conditions. The study was conducted in accordance with the International Ethical Norms on Animal Care and Use as contained in NIH publication/80-23, revised in 2010.

Bonny Light Crude Oil (BLCO): BLCO was obtained from the Nigerian National Petroleum Corporation (NNPC) Warri, Nigeria**.** A daily oral dose 0.75ml of BLCO/Kg bwt/day was administered to the treatment group.

Experimental Protocol: In total, 10 female Wistar rats (10 weeks; 150–170 g) with normal estrous cycle were used. The estrous cycle of the rats was monitored daily according to the method described by Marcondes *et al*. (2002). Rats in proestrous were mated with proven male breeder at a ratio of 1:1 overnight and the presence of sperm in their vaginal or copulatory plug in the next morning marked gestational day (GD 1). After pregnancy had been confirmed, animals were randomly assigned into two groups of five animals each and treated accordingly during gestation. Administration was carried out through oral gavage between 8 am and 10 am daily at gestation days 1-21. Group 1 was administered 0.75ml of distilled water/kg bwt/day (control). Group 2 was administered 0.75ml of BLCO/Kg bwt/day. The litter size was standardized to six pups per litter. After delivery, the following parameters were measured: body weight at birth, postnatal day (PND) 21 (at end of weaning) and PND 90 (at adulthood). Malondialdehyde (MDA) levels, catalase and superoxide dismutase (SOD) activities in the homogenate of the testis and epididymis (Male)/Ovary and uterus (Female) were also assessed at PND 90. Serum testosterone, LH and FSH levels as well as the sperm indices (Motility, Morphology, and Counts) were assessed in the male offspring. Serum estradiol, LH and FSH, estrous cycle length and frequency were assessed in female offspring. Pubertal timing was also assessed in both male and female offsprings.

Serum and Tissue Collection: Blood samples were collected from the orbital sinus of male and female offsprings under Sodium thiopentone anaesthesia (50 mg/kg, i.p.) at PND 90 days into polythene tubes and allowed to clot for 1 h. The blood samples were then centrifuged at 3000 rpm for 10 min. Serum was aspirated and stored at 4°C.

After blood sample collection, the rats were carefully sacrificed by cervical dislocation. During dissection, the testes and epididymis in male/ovary and uterus in females were carefully collected and rinsed in ice-cold 1.15% KCl solution. Dry weights of the tissues were recorded. They were thereafter placed in 0.1 M potassium phosphate buffer pH 6.5 and homogenized using a homogenizer. The homogenate was centrifuged in a cold centrifuge at 10,000 rpm for 10 min. The supernatant was removed and stored in a refrigerator (at about 4◦C) for analysis of oxidative stress. Biochemical analysis was done within 48 hrs of sample collection.

Biochemical Assays

Determination of Oxidative Stress (Lipid Peroxidation Assessment): Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation. This was carried out according to the methods described by Buege and Aust (1978).

Determination of Catalase Activity: Catalase activity was determined according to the method of Claiborne (1985). The method is based on the loss of absorbance observed at 240 nm as catalase splits hydrogen peroxide. Despite the fact that hydrogen peroxide has no absorbance maximum at this wavelength, its absorbance correlates well enough with concentration to allow its use for a quantitative assay. An extinction coefficient of 0.0436 mM-1cm-1 was used.

Determination of Superoxide Dismutase (SOD) Activity.: SOD activity was evaluated according to methods of Misra and Fridovich (1972). The ability of superoxide dismutase to inhibit the autooxidation of adrenaline at pH 10.2 makes this reaction a basis for the SOD assay. Superoxide anion (O_2) generated by the xanthine oxidase reaction is known to cause the oxidation of adrenaline to adrenochrome. The yield of adrenochrome produced per superoxide anion increased with increasing pH and also with increasing concentration of adrenaline. These led to the proposal that autooxidation of adrenaline proceeds by at least two distinct pathways, one of which is a free radical chain reaction involving superoxide radicals, and hence could be inhibited by SOD.

Determination of Tissue Protein Level: Protein estimation was done by method of Lowry *et al*. (1951). The Folin-Ciocalteau reagent was used in the quantification of proteins by Lowry. In its simplest form, the reagent detects tyrosine residues due to their phenolic nature. The reaction of a protein in solution with the Folin reagent occurs in two stages: Reaction with Cu^{++} in alkaline medium and reduction of the phosphomolybdic-phosphotungstic reagent by the Cu^{++-} protein complex. The reduced complex gives a blue solution with an absorption in the red portion of the visible spectrum (600–800 nm).

Determination of anogenital distance: Anogenital distance (AGD) at birth was determined by using a digital Vernier calliper to measure the distance between the posterior base of the sex papilla and the anterior anus at PND 1.

Detecting Testes Descent: The rats were studied daily and the days testes descent was noticed and recorded (Ostby and Gray, 2004).

Detecting Puberty in Male and Female Offsprings: To detect the periputial separation (PPS), male rats were checked daily beginning after testis descent was noticed in the rat to ensure no rats have periputial separation. This was done by applying gentle pressure to the prepuce to retract the prepuce and expose the glans penis. PPS is complete when the entire perimeter of the prepuce can be retracted evenly around the base of the glans penis (Ostby and Gray, 2004). The day of vaginal opening was recorded and taken as the onset of puberty in female (Ostby and Gray, 2004).

Determination of Sperm Indices: Sperm analysis was done by microscopy as previously described (Raji and

Bolarinwa, 1997; Raji *et al*., 2003). Epididymal spermatozoa were obtained by mincing the epididymis with anatomical scissors in 5ml of pre-warmed physiological saline and incubated for 2 min. An aliquot of this solution was placed in improved Neubauer counting hemocytometer and motile sperm were counted by using microscope at 400× magnification. Non-motile sperm numbers were first determined, followed by counting of total sperm. Sperm motility was expressed as a percentage of motile sperm of the total sperm counted. Percentage of morphologically abnormal spermatozoa was determined by preparing two slides with Hemaoxylin and Eosin stains for morphological examination of live–dead ratio. A total of 400 sperm cells were counted on each slide under light microscope at 400× magnifications. Sperms with abnormal head and/or tail were considered abnormal. Sperm motility, viability and count were done immediately and quickly. A sperm viability test was done using eosin/negrosin stain (containing 1 g of Eosin and 4 g of Negrosin in 100 ml phosphate buffer). A drop of the epididymal fluid was placed on the slide and two drops of the stain was added. A thick smear was made from this and dried. After this, the slide was studied under light microscope using 40x objective lens. The unstained spermatic cells were considered as live sperms while the stained ones was considered as dead sperm. A minimum of 100 spermatic cells (both stained and unstained) was counted and an average was taken for the percentage live sperm.

Determination of Estrous cycle

Vagina cytology: Using Marcondes technique (Marcondes *et al*., 2002), about 0.1ml of 0.9% normal saline solution was gently introduced 2-3 times into the vagina of the rat to produce a vaginal lavage. The pipette was withdrawn and its content was smeared on a microscope slide and viewed using x40 magnification lens of the microscope. Estrous cycle lasted about 4-5days extending from the day of proestrus characterized by the presence of nucleated vaginal epithelial cells followed by the estrus phase presenting cornified vaginal mucosa cells, metestrus, was a combination of the cells and the diestrus phase, characterized by the presence of leukocytes in the vaginal smear.

At the end of the experiments animals of control and BLCO treated groups were euthanised.

Statistical analysis: Data were expressed as means ± S.E.M. Statistical comparisons were performed using independent student t-test. Differences between the treatment groups with a P-value < 0.05 were considered significant. Data were analyzed with the use of GraphPad Prism software version 8.02® (LA Jolla, CA, USA).

RESULTS

Effects of maternal exposure to BLCO during gestation on birth weight anogenital distance, testis descent and preputial separation/vaginal opening of the offspring of Wistar rats: There was a significant reduction (P<0.05) in birth weight and anogenital distance (AGD) in the male and female offspring of rats exposed to BLCO during gestation (Table 1) when compared with the control. There was also a significant increase (P<0.05) in the days of testis descent and preputial separation in the male offspring as well as vaginal opening in the offspring of rats exposed to BLCO during gestation relative to the control (Table 2).

Table 1:
The morphometric indices of the male offspring following maternal treatment with BLCO during gestation

Group/	Birth weight (g)	AGD at Birth (cm)	Preputial separation (days)	Testis descent (days)
Control	7.78 ±0.03	4.21 ±0.01	53.00 ±0.78	17.00 ±1.21
Treatment (BLCO)	4.98 ±0.00*	3.31 ±0.03*	65.60 ±0.40*	23.00 ±1.01*

**the mean is statistically significant in the treatment group when compared with the control*

Table 2:
The morphometric indices of the female offspring following maternal treatment with BLCO during gestation

Group/	Birth weight (g)	AGD at Birth (cm)	Vaginal opening	Estrous cycle length (days)
Control	7.58 ±0.32	2.34 ±0.01	47.00± 0.575	4.700 ± 0.03
Treatment (BLCO)	5.72 ±0.31*	1.82 ±0.01*	55.60 ±0.43*	4.50 ±0.04

*the mean is statistically significant in the treatment group when compared with the control

Effects of maternal exposure to BLCO during gestation on relative testicular and epididymal weight of the Male offspring of Wistar rats: Maternal exposure to BLCO during gestation significantly raised (P<0.05) the relative testis, epididymal, ovary and uterine weight in the offspring of Wistar rats when compared with the control (Figure 1 and 2).

Effects of maternal exposure to BLCO during gestation on sperm indices (count, motility and morphology) of the male offspring of Wistar rats: There was a significant reduction in sperm count and sperm motility of the male offspring of Wistar rats exposed to BLCO during gestation. However, percentage abnormal morphology sperm cells was significantly raised in the group exposed to BLCO during gestation when compared with the control group (Figure 2, 3 and 4).

Effects of maternal exposure to BLCO during gestation on length of estrous cycle of the female offspring of Wistar rats: There was no significant difference in the overall length of the cycle, length of proestrus and estrous phase in the female offspring of the BLCO treated rats and control. However, the length of metestrous was raised while that of diestrous was reduced (p<0.05).

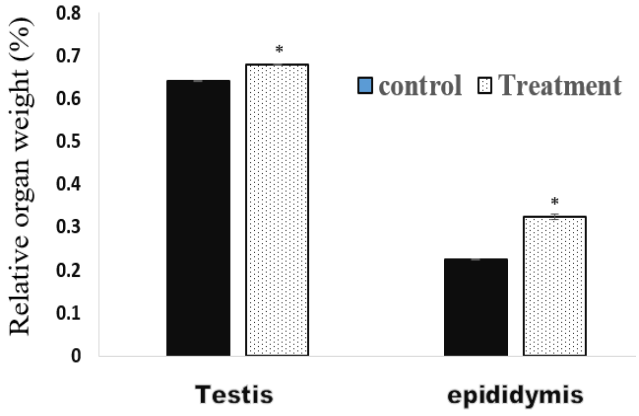

Figure 1:
Relative organ weight of the male offspring of Wistar rats following maternal exposure to BLCO during gestation in Wistar rats
**Mean is statistically significant in the treatment group when compared with the control*

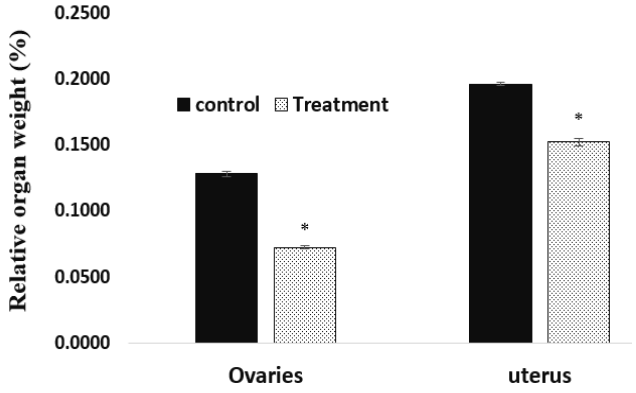

Figure 2:
Relative organ weight of the female offspring of Wistar rats following maternal exposure to BLCO during gestation in Wistar rats
**Mean is statistically significant in the treatment group when compared with the control*

Effects of maternal exposure to BLCO during gestation on serum hormonal profile of the Male (FSH, LH and Testosterone) and female (estradiol, FSH and LH) offsprings of Wistar rats: Serum testosterone, was significantly reduced in the male offspring of BLCO group at 12 weeks of postnatal life relative to the control. However, FSH level was significantly raised ($p<0.05$) in the male offspring of BLCO treated group when compared with the control. In the female offspring, there was a significant increase in the serum estradiol and FSH level. Meanwhile serum LH level was significantly reduced ($p<0.05$) at 12 weeks of postnatal life in the female offspring of BLCO group when compared with the control.

Table 3:
The sperm indices of the male offspring following maternal treatment with BLCO during gestation

Group/	Sperm count (10^6/ml)	Sperm Motility (%)	Sperm Morphology (Abnormal) (%)
Control	185.10 ±4.31	84.00 ±2.12	47.00 ±0.70
Treatment (BLCO)	174.20 ±2.90*	62.00 ±1.930*	67.40 ±0.61*

**Mean is statistically significant in the treatment group when compared with the control*

Table 4:
The estrous cycle length of the female offspring following maternal treatment with BLCO during gestation

Group/	Proestrous (%)	Estrous (%)	Diestrous (%)	Metestrous (%)
Control	13.93 ± 1.21	14.42 ± 0.04	53.20 ± 0.24	17.90 ±0.09
Treatment (BLCO)	13.00 ±0.92	14.50 ±0.36	48.40± 0.29*	24.50± 0.72*

*the mean is statistically significant in the treatment group when compared with the control

Effects of maternal exposure to BLCO during gestation on indices of oxidative stress in the Male (testes and epididymis) and female (ovaries and uterus) offspring of Wistar rats: The level of malondialdehyde (MDA) was significantly raised in the ovaries and uterus of the BLCO treated female offsprings of Wistar rats as compared to the control. Catalase activity was significantly decreased in the ovaries and uterus of the BLCO treated female offspring of wistar rats relative to the control. The level of superoxide dismutase (SOD) was significantly lower in the ovaries and uterus of the BLCO treated female offsprings of Wistar rats relative to the control. The testicular and epididymal MDA was significantly raised in the male offsprings. The SOD and Catalase activities were significantly reduced in the testis and epididymis of the male offsprings exposed to BLCO during gestation.

Table 5:
The serum hormonal profile of offspring following maternal treatment with BLCO during gestation

	Male			Female		
Group/	Testosterone (nmol/l)	FSH (mlU/ml)	LH (mlU/ml)	Estradiol (pg/ml)	FSH (mlU/ml)	LH (mlU/ml)
Control	0.69± 0.02	0.35± 0.002	0.26± 0.00	15.55±0.39	0.59± 0.05	0.52± 0.31
Treatment (BLCO)	0.57± 0.01*	0.51± 0.01*	0.25± 0.00	17.92±0.37*	0.92± 0.03*	0.09± 0.00*

*the mean is statistically significant in the treatment group when compared with the control

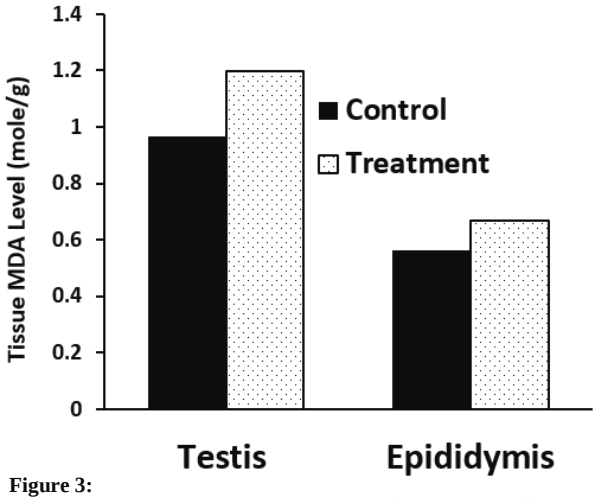

Figure 3:
Tissue Malondialdehyde (MDA) level of the male offsprings following maternal exposure to BLCO during gestation in Wistar rats
**Mean is statistically significant in the treatment group when compared with the control*

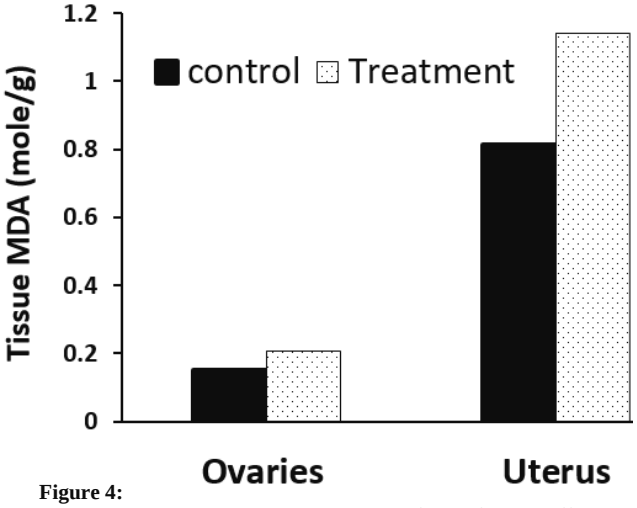

Figure 4:
Tissue Malondialdehyde (MDA) level of the female offspring following maternal exposure to BLCO during gestation in wistar rats
**Mean is statistically significant in the treatment group when compared with the control*

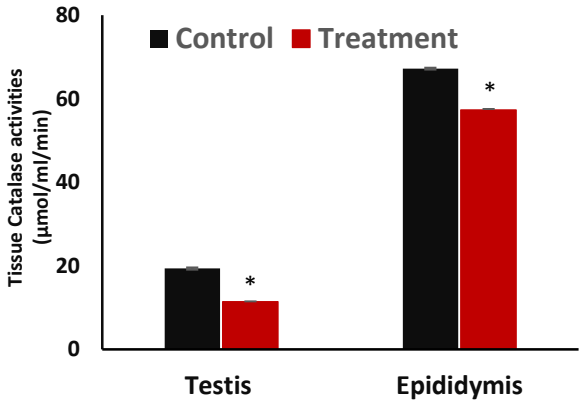

Figure 5:
Tissue Catalase activities of the male offspring of Wistar rats following maternal exposure to BLCO during gestation in Wistar rats
**Mean is statistically significant in the treatment group when compared with the control*

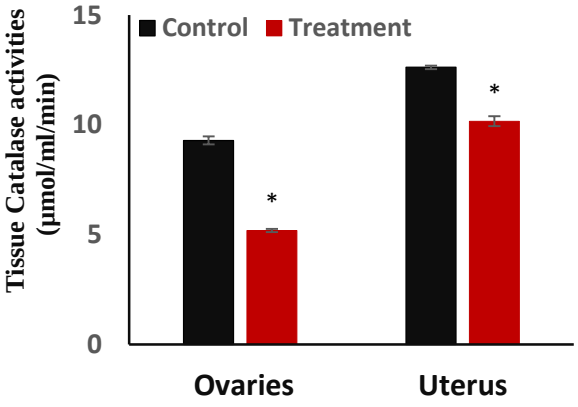

Figure 6:
Tissue Catalase activities of the female offspring of Wistar rats following maternal exposure to BLCO during gestation in Wistar rats.
**Mean is statistically significant in the treatment group when compared with the control*

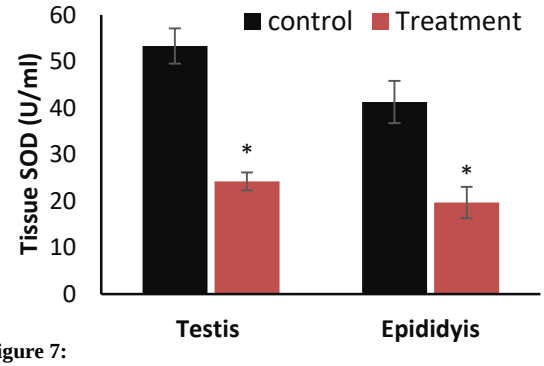

Figure 7:
Tissue Superoxide Dismutase (SOD) activities of the male offspring of Wistar rats following maternal exposure to BLCO during gestation in Wistar rats
**Mean is statistically significant in the treatment group when compared with the control*

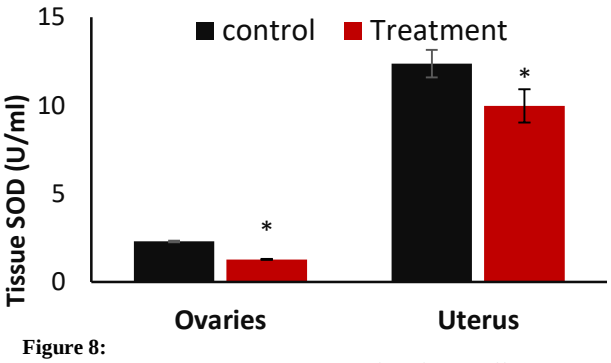

Figure 8:
Tissue Superoxide Dismutase activities of the female offspring of Wistar rats following maternal exposure to BLCO during gestation in Wistar rats
**Mean is statistically significant in the treatment group when compared with the control*

DISCUSSION

Several environmental pollutants have been reported to interfere with the endocrine system. Many of these endocrine disruptors are released into the environment due to several human activities such as industrial and manufacturing activities, mining and oil exploration activities (Darbre, 2018). In this study, exposure to BLCO during gestation significantly reduced birth weight, relative ovarian weight and uterine weight of the BLCO treated

female offspring of Wistar rats. The decrease in the F1 offspring body weight and relative organ weight may be a pointer to reduction in the growth and development of reproductive organs (Tutian *et al*., 2008). Barker's hypothesis postulates that an infant's birth weight (BW) is influenced by the intrauterine environment, and an adverse intrauterine environment altered the expression of certain genes that control the development and function of organs and tissues (De Boo & Harding, 2006). In agreement with this observation BLCO and its constituent hydrocarbon (such as Polycyclic Aromatic Hydrocarbon (PAF) have been previously reported to induce developmental malformation in the offsprings (Feuston and Hamilton, 1997; Fischer *et al*., 2007).

Disorders in sex steroid balance during fetal development generally affects the reproductive system development (Sharpe, 2001). Reduction in proportion of androgen to estrogen level and exposure to other anti-androgenic agents induced gross alteration in male reproductive structures and functions from fetal life (Sharpe, 2001; Rivas *et al*., 2002; Welsh *et al*., 2008). In agreement with this observation, sperm indices, anogenital distance at birth and serum testosterone level were reduced in the male offspring following maternal treatment with BLCO during gestation in this study. In addition, pubertal timing, testis descent was also delayed. These indices are generally known to be androgen dependent (Ostby and Gray, 2004; Jeje and Raji, 2017). These suggest that BLCO exposure during gestation may affect the androgen production and or released from the testis. If the alteration in testosterone level is centrally mediated required further assessment. We observed that serum LH level was not significantly influenced by the low testosterone in the male offspring. Normally, testosterone released is regulated through a negative feedback mechanism that release LH from the anterior pituitary gland (Aron *et al*., 2007). Disruption in the negative feedback mechanism could therefore affect reproductive functions (Aron *et al*., 2007).

Irregularity in estrous cycle was observed in the female offspring of treated rat's, however, there was no statistical difference in the length of estrous cycle of treated rat F1 offspring and the control F1 offspring. This is in agreement with Raji and Hart (2012), who also reported no significant difference in length of estrous cycle following pre and post treatment with BLCO at different doses in female Wistar rats. This indicates that BLCO sub-lethal administration might not interfere in oestrous cycle and ovarian cycle activities of not just the rats exposed but also in the F1 female offspring. Conversely, there were changes at each phase of the cycle, length of estrus and proestrus were insignificantly different but there was a significant increase in metestrus phase and a significant decrease in diestrus phase.

The increase in metestrus phase indicates the availability of matured Graafian follicles and maturation of secondary follicles, suggesting that ovulation was inhibited since length of estrus phase was not significantly different (Shrestha *et al*., 2010). Diestrus is characterized by the elevation in circulating progesterone from the corpus luteum that rises shortly after ovulation and it persist until luteolysis occurs because at the diestrus phase, the corpus luteum now actively secretes progesterone. Therefore, a shorter diestrus length in the treated rats offspring may further worsen distorted ovarian cycle, consequently reducing oocyte number that may ultimately reduce fertility and number of viable offspring (Raji, and Hart., 2012).

Reports from several studies have implicated oxidative stress in the pathogenesis of infertility (Ola-Mudathir *et al*., 2008). Oxidative stress is linked with high level of reactive oxygen species which results in lipid peroxidation of the spermatozoa outer membrane. This results in loss of motility (Urata *et al*., 2001), decrease sperm-oocyte fusion capacity and increased cell destruction due to chromatin damage (Aitken, 1994; Aitken and Krausz, 2001). Therefore, for the protection of reproductive system from oxidative stress, the testis, epididymis and spermatozoa have the capacity to produce antioxidant enzymes such as catalase, SOD, glutathione reductase (Tramer *et al*., 1998; Zubkova and Robaire; 2004). The result from this study suggests a significant reduction in the antioxidant enzyme SOD and catalase with an increased in the level of by-product of lipid peroxidation (MDA) in the reproductive structures of male and female offsprings. This suggests the possibility of an increase exposure to oxidative damage in the reproductive structures following maternal exposure to BLCO during gestation.

In conclusion, maternal exposure to bonny light crude oil during gestation may induced alterations in reproductive functions in the male and female offspring. In addition, the reproductive structures may be more susceptible to oxidative stress.

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