

DNA fragmentation and oxidative stress compromise sperm motility and survival in late pregnancy exposure to omega-9 fatty acid in rats

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ARTICLE INFO	ABSTRACT
Article type: Original article	Objective(s): The aim of this study was to evaluate the oxidative status and DNA integrity in testes of wistar rat offspring exposed to omega-9 monounsaturated (MUFA) at different times of late organogenesis.
Article history: Received: Sep 14, 2015 Accepted: Feb 4, 2016	Materials and Methods: Sixty female rats were divided into six groups of 10 animals. The first group served as control and received the drug vehicle, olive oil (1 ml/kg/day). The second, third, fourth, fifth and sixth group received 1000 mg/kg of oleic acid on gestation day 15 (D15), 16 (D16), 17 (D17), 18 (D18) and 19 (D19), respectively. Male pups were allowed to attain puberty and thereafter, blood was taken for hormonal analyses. Sperm count and motility were assessed. Testes homogenate was used for the determination of biochemical variables. Testes DNA was also determined.
Keywords: Deoxyribonucleic acid Oleic acid Oxidative stress Superoxide dismutase Total protein	Results: The results showed that sperm count and motility were significantly decreased in the treated groups as compared to the control. There was a marked increase in the malondialdehyde level in rat testes from all of the treated groups as compared to the control ($P<0.05$). DNA from the testes of rats of D19 had the highest level of fragmentation as compared to the control. Conclusion: Omega-9 MUFA exposure <i>in utero</i> imposes negative effects on sperm variables and increases the level of sperm DNA fragmentation and oxidative stress.

► Please cite this article as:

Oluwakemi O, Olufeyisipe A. DNA fragmentation and oxidative stress compromise sperm motility and survival in late pregnancy exposure to omega-9 fatty acid in rats. Iran J Basic Med Sci 2016; 19:511-520.

Introduction

Omega-9 (oleic acid) is a monounsaturated fatty acid found in animal and vegetable oils. It is also found in olives, avocados, almonds, pistachio nuts, cashew nuts, hazelnuts, macadamia nuts and papaya seeds (1, 2). Oleic acid lowers the risk of heart attack as well as atherosclerosis. It also helps to prevent cancer (2).

A considerable body of evidence has indicated that dietary fat composition influences male reproductive function by modifying the cholesterol-phospholipid composition of testicular plasma membranes (3). The three types of natural fatty acids (FAs) namely, saturated, monounsaturated (MUFA) and polyunsaturated (PUFA) play various roles in male reproduction. For instance, several studies in men (4) and in boars (5, 6) have shown that omega-3 PUFA are beneficial to the male reproductive capacity; although other studies in humans (7) as well as boars (8) did not show any effect for omega-3 PUFAs. Scientific research in the last 100 years have also reported that omega-6 PUFAs in Western diets have intensely increased and this has also increased

the omega-6 /omega-3 ratio of 25:1 to 40:1 (9) which has also been attributed to the impaired semen quality from oligozoospermic men and/or asthenozoospermic men (10).

Recently, we reported that exposure of male offspring to omega-9 during pregnancy altered the male reproductive hormones, reduced the weight of reproductive organs, altered the age of sexual maturation as well as epididymal functions (11). Investigations have also shown that the testis has a unique immune structure that helps in the maintenance of spermatogenesis (12). When reactive oxygen species (ROS) are excessively produced, there is a disturbance in the balance of pro-oxidative and antioxidative factors. Thus, if the amounts of ROS exceed the antioxidant capacity, peroxidation-induced damage to the spermatozoa is inevitable (13). Scientific evidence reveals that reduced antioxidant capacity of seminal plasma is sufficient to ensure abnormalities to the sperm (13, 14). Disturbances such as peroxidation, germ cell apoptosis and alteration of sperm motility, morphology and count have been reported in infertile men (15) although the causes

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and molecular mechanisms of oxidative stress are yet to be elucidated. What is known is that DNA damage may be a trigger for such processes (12). Thus, testicular DNA integrity and its effect on normal spermatogenesis have been of great interest in the last couple of years (13).

The effect of *in utero* exposure of male offspring to omega-9 MUFA during late organogenesis has not been sufficiently investigated. The purpose of this study was to evaluate the involvement of oxidative processes and DNA integrity of male offspring exposed to omega-9 MUFA on different days of late organogenesis. For this purpose, in our study, lipid peroxidation, redox potentials, LDH-x isoenzyme activity, cellular protein markers and DNA activity of the testes were checked.

Materials and Methods

Animals

All experimental protocols were conducted in accordance with the internationally accepted guidelines for laboratory animal use and care as mentioned in the Helsinki's Declaration. The experiments reported in this study were approved by our Institutional Ethics Committee. The experiment was carried out in non-pregnant sexually mature Wistar rats weighing approximately 190-220 g, obtained from the Central Animal House of the College of Medicine of the University of Lagos, Nigeria. Also, sexually mature male rats weighing between 300-350 g were also obtained. These animals were housed in clear polypropylene cages lined with wood chip beddings in an airy, temperature-controlled (21-23 °C) and light-controlled (12 hr light: 12 hr dark) animal house. Animals were acclimatized two weeks prior to being used in experimental procedures. Animals were fed with commercial standard pellet diet (Livestock Feeds, Lagos, Nigeria) and water *ad libitum*.

Drugs, doses and design

Oleic acid (Sigma Chemical Co. USA) was used in this study and administered at an LD₅₀ of 1000 mg/kg. Male rats were housed singly in the cages. For the purpose of mating, two female rats at pro-oestrus were placed in a cage of one male overnight in the ratio 2:1. The following morning, mating was confirmed if spermatozoa were seen and the day was regarded as the first day of gestation (GD 1) (16). Sixty female rats were divided into six groups of 10 animals each. All chemicals were orally administered using intragastric tubes. The first group served as control and received the drug vehicle, olive oil at 1 ml/kg/day. The second, third, fourth, fifth and sixth group received a dose of 1000 mg/kg of oleic acid on gestation day 15 (D15), gestation day 16 (D16), gestation day 17 (D17), gestation day 18 (D18) and gestation day 19 (D19),

respectively. On gestation D20, pregnant rats were transferred to bedding material and allowed to give birth. Culling was introduced. Thus, each female rat in each group was allowed to nurse 9 male pups throughout the lactation period. This was to eliminate the effect of undernutrition or overnutrition in the pups. Male pups were allowed to attain puberty. Twenty-four hours after attainment of puberty, blood samples were directly collected from the heart by means of heparinised syringes. Blood was taken for hormonal analysis. The cauda epididymis from each rat was dissected, weighed, and used for assessment of sperm count and motility. Testes were weighed and cut into small pieces and homogenized in ice-cold 0.15 M KCl to give 10% homogenate. The homogenate was then divided into several aliquots and used for biochemical analysis (17).

Sperm analyses

Sperm count and motility were determined according to the method described by Anderson *et al* (18). Spermatozoa were obtained by making small cuts into the cauda epididymis from each rat. Spermatozoa were then suspended carefully in 20 ml Krebs-Ringer bicarbonate buffer pre-warmed to 37 °C. Afterwards, 10 µl of the suspension was transferred to each chamber of a Neubauer hemocytometer (Germany) and overfilling or underfilling was avoided. Separate counts for motile and non-motile sperms in each chamber were done. Motility of spermatozoa was microscopically examined directly in aliquot of sperm suspension and scored as percentage of total sperms (18).

Determination of lactic dehydrogenase isoenzyme (LDH-X) activity

Lactate dehydrogenase (LDH-X) activity was determined by the method of Cheever *et al* (19). The testes homogenate was centrifuged at 1000 g for 30 min, and the supernatant was filtered through 0.45-µm pore-size Acrodisc (USA). The filtrate was used as LDH-X enzyme preparation. Quartz cuvettes containing 3 ml of the specific substrate (106 mM trizma, 60 mmol DL-alpha hydroxy caproic acid + 0.05% sodium azide + 0.9 Mm NAD⁺) were incubated with 20 µl of the enzyme preparation for 10 min. The temperature of the reaction mixture was kept at 30 °C, and the change in the absorbance was measured spectrophotometrically at 340 nm using Shimadzu Spectrophotometer UV 1201 (Japan). The LDH-X activity was calculated as international unit per gram wet tissue.

Determination of antioxidant activities

The activity of the superoxide dismutase (SOD) enzyme in the testis homogenate was determined

according to the method described by Sun and Zigman (20). The reaction was carried out in 0.05 M sodium carbonate buffer, pH 10.3, and was initiated by the addition of epinephrine in 0.005 N HCl. The catalase (CAT) activity was determined by measuring the exponential disappearance of H₂O₂ at 240 nm and expressed as units/mg of protein as described by Aebi (21). The reduced glutathione (GSH) content of the testis homogenate was determined using the method described by Van Dooran *et al* (22). The GSH determination method is based on the reaction of Ellman's reagent 5, 5'-dithiobis-2-nitrobenzoic acid (DNTB) with the thiol group of GSH at pH 8.0 to produce 5-thiol-2-nitrobenzoate, which is yellow at 412 nm. Absorbance was recorded using Agilent UV-Visible Spectrophotometer in all measurements.

Determination of malondialdehyde (MDA) level

As a marker of lipid peroxidation, the level of malondialdehyde (MDA) in the testis homogenate was measured by the method of Uchiyama and Mihara (23) based on production of thiobarbituric acid reactive substances (TBARS). The development of a pink complex with maximum absorption at 535 nm was taken as an index of lipid peroxidation.

Determination of protein content

Testicular protein content was determined according to the method of Lowry *et al* (24) using bovine serum albumin as a standard.

Determination of cholesterol level

Testicular cholesterol level was determined according to the method of Kim and Goldner (25).

Determination of hormonal levels

Testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in rat serum were measured by enzyme immunoassays using commercially available kits (Sigma, USA). Samples were measured in the same assay to avoid inter-assay variations.

Determination of testicular DNA

Testis DNA was extracted by phenol/chloroform/isoamyl alcohol (26). Briefly, 3 ml of previously prepared testis homogenate was still down by centrifugation at 1,000 rpm for five min then, washed with phosphate buffered saline (PBS) pH 7.4. Then, 2 ml of Tris-EDTA (TE) buffer [1 M Tris-HCl pH

8 (100 ml) and 0.5M EDTA (100 ml)] was added to the pellet, mixed and completed to 300 ml with distilled water. Afterwards, 100 µl proteinase K (10 mg/ml) and 240 µl 10% SDS (sodium dodecylsulphate) were added, shaken gently and incubated at 45 °C in water bath, overnight. The following day, 2.4 ml equilibrated phenol was added, shaken and centrifuged at 3,000 rpm for 10 min. The supernatant was transferred to a new tube and 1.2 ml of phenol then 1.2 ml of chloroform/isoamyl alcohol (24:1) were added, shaken for 5–10 min and centrifuged at 3,000 rpm for 10 min. The supernatant was transferred to a new tube and 2.4 ml of chloroform/isoamyl alcohol (24:1) was added, shaken for 5–10 min, and centrifuged at 3,000 rpm for 5–10 min. Next, 25 µl of sodium acetate (3 M, pH 5.2) and 5 ml of cold absolute ethanol were added to the supernatant and shaken gently which resulted in DNA precipitation. The DNA was hooked out and washed with ethanol and afterwards dissolved in TE buffer and the concentration was obtained by determination of the absorbance at 260 nm. The purity of the extracted DNA was determined by assessment of the ratio of the absorbance at 260/280. The purity of extracted DNA was above 95%. The extracted DNA was digested by DNase-1 (1 U/1 µg DNA). Digested DNA was subjected to determination of 8-HDG according to the protocol of the commercially available Kit by ELIZA assay (BIOXYTECH, 8-HDG-EIA Kit, OXIS, Health Product. Inc., 6040 N Cutter Circle, Suite 317 Portland, OR 97217–3935 USA).

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Statistical comparison between different groups were done by using Graph Pad Prism 4 software through one way analysis of variance (ANOVA) followed by Tukey-Kramer for a multiple comparisons test. The significance level was accepted at $P < 0.05$.

Results

Sperm count and motility as markers for normal testicular function

The data showed that the sperm count and motility were significantly reduced in the D16, D17, D18 and D19 groups respectively as compared to the control group as shown in Table 1.

Table 1. Effect of late pregnancy exposure to omega-9 monounsaturated fatty acid on sperm indices in rats

	Control	D15	D16	D17	D18	D19
Count ($\times 10^6$ /ml)	98.60 ± 8.70	55.27 ± 3.51 ^a	50.11 ± 2.48 ^a	48.9 ± 1.33 ^a	45.7 ± 2.05 ^a	40.5 ± 2.25 ^a
Motility (%)	80.8 ± 7.27	51.6 ± 4.25 ^a	50.4 ± 3.75 ^a	45.3 ± 2.78 ^a	40.0 ± 2.00 ^a	31.3 ± 0.55 ^a

Values are expressed as mean ± SEM (n=54), ^a $P < 0.05$ vs. control, ^b $P < 0.05$ vs. D15, ^c $P < 0.05$ vs. D16, ^d $P < 0.05$ vs. D17, ^e $P < 0.05$ vs. D18. One-way ANOVA followed by Tukey-Kramer for a multiple comparisons test. Day 15= D15, day 16= D16, day 17= D17, day 18= D18, day 19= D19

Table 2. Effect of late pregnancy exposure to omega-9 monounsaturated fatty acid on sperm malondialdehyde and antioxidant levels in rats

	Control	D15	D16	D17	D18	D19
MDA(nmol/ml)	0.26 ± 0.02	0.36 ± 0.02 ^a	0.34 ± 0.01 ^a	0.33 ± 0.00 ^a	0.35 ± 0.01 ^a	0.37 ± 0.02 ^a
GSH (μmol/ml)	0.34 ± 0.01	0.21 ± 0.02 ^a	0.21 ± 0.02 ^a	0.30 ± 0.01 ^{a,b,c}	0.24 ± 0.01 ^a	0.26 ± 0.02 ^a
SOD (μmol/ml)	0.003 ± 0.00	0.003 ± 0.00	0.001 ± 0.00 ^{a,b}	0.003 ± 0.00 ^c	0.002 ± 0.00 ^{a,b,c,d}	0.001 ± 0.00 ^{a,b,d}
CAT (mmol/ml)	0.14 ± 0.00	0.17 ± 0.00 ^a	0.18 ± 0.00 ^{a,b}	0.17 ± 0.00 ^{a,c}	0.17 ± 0.00 ^{a,c}	0.17 ± 0.00 ^{a,c}

Values are expressed as mean ± SEM (n=54), ^aP<0.05 vs. control, ^bP<0.05 vs. D15, ^cP<0.05 vs. D16, ^dP<0.05 vs. D17, ^eP<0.05 vs. D18. One-way ANOVA followed by Tukey-Kramer was used for multiple comparisons test. Day 15= D15, day 16= D16, day 17= D17, day 18= D18, day 19= D19

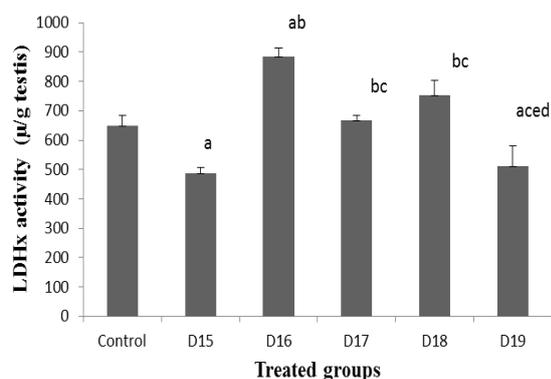


Figure 1. Effect of late pregnancy exposure to omega-9 monounsaturated fatty acid on testicular lactic dehydrogenase isoenzyme activity in rats. Values are expressed as mean ± SEM (n=54), ^aP<0.05 vs. Control, ^bP<0.05 vs. D15, ^cP<0.05 vs. D16, ^dP<0.05 vs. D17, ^eP<0.05 vs. D18. One-way ANOVA followed by Tukey-Kramer was used for multiple comparisons test. Day 15= D15, day 16= D16, day 17= D17, day 18= D18, day 19= D19

Testicular LDH-x isoenzyme activity as a marker of normal spermatozoal metabolism and testicular toxicity

The activity of LDH-x enzyme in the testis of the rats was significantly reduced in the D15 and D19 groups as compared to the control, although it was increased in the D16 group as compared to the control. Also, a significant reduction was seen in D19 as compared to the D16, in D19 as compared to D18, in D19 as compared to D17, in D17 as compared to D16, in D18 as compared to D16 groups. A significant increase was observed in D16 as compared to D15, in D17 as compared to D15 and in D18 as compared to D15 groups.

Testicular GSH content as a marker of redox potential

The testicular GSH values of rats from D16, D17, D18 and D19 groups were significantly reduced as compared to the control group (Table 2).

Testicular SOD content as a marker of redox process

The testicular SOD activities of rats from D16, D18, and D19 groups were significantly reduced ($P<0.05$) as compared to the control group. There were also significant decreases among treated groups and an increase in the D17 as compared to D16 as well as D18 as compared to D16 groups (Table 2).

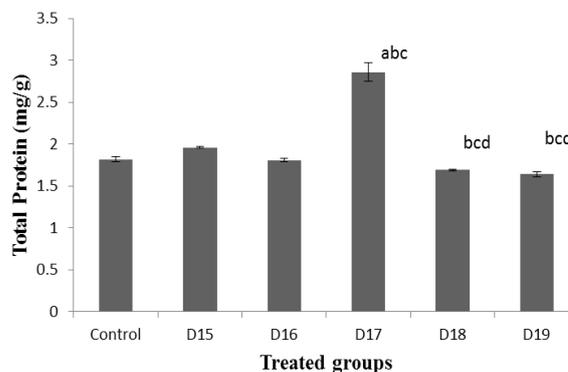


Figure 2. Effect of late pregnancy exposure to omega-9 monounsaturated fatty acid on testicular total protein in rats. Values are expressed as mean ± SEM (n=54), ^aP<0.05 vs. control, ^bP<0.05 vs. D15, ^cP<0.05 vs. D16, ^dP<0.05 vs. D17, ^eP<0.05 vs. D18. One-way ANOVA followed by Tukey-Kramer was used for multiple comparisons test. Day 15= D15, day 16= D16, day 17= D17, day 18= D18, day 19= D19

Testicular CAT content as a marker of redox process

The testicular CAT activities of rats from D16, D17, D18 and D19 groups significantly increased as compared to the control (Table 2).

Testicular MDA content as a marker of lipid peroxides formation

There was a significant increase in the MDA level in rat testes from D16, D17, D18 and D19 groups as compared to the control (Table 2).

Testicular protein as a marker of cellular protein and androgen activity

There was a significant increase in the testicular protein of rats from D17 as compared to the control. Significant reductions were observed in the treated rats from D18 as compared to D15, in D19 as compared to D15, D18 as compared to D16, in D19 as compared to D16, and in D19 as compared to D17 groups. There was also a significant ($P<0.05$) increase in the testicular protein of rats from D17 as compared to D15 and in D17 as compared to D16 groups.

The total protein (TP) level of rats from D16 group showed significant ($r = 0.11$, $P<0.05$) positive correlation for LDH. TP level of rats from D18 group revealed significant ($r= 0.98$, $P<0.05$) positive correlation for LDH. Also, TP level of rats from D19

	Control	D15	D16	D17	D18	D19
LH (u/l)	0.34 ± 0.05	2.37 ± 0.16	0.09 ± 0.01	0.45 ± 0.01	5.36 ± 0.48 ^{a,b,c}	5.53 ± 0.25 ^{a,b,c}
FSH (u/l)	1.19 ± 0.06	1.17 ± 0.57 ^a	0.45 ± 0.03 ^b	0.33 ± 0.00 ^b	3.52 ± 0.46 ^{a,c,d}	3.08 ± 0.27 ^{a,c,d,e}
Testosterone (ng/ml)	4.10 ± 0.02	4.25 ± 0.16	4.09 ± 0.12	7.41 ± 0.06 ^{a,b,c}	2.72 ± 0.05 ^{a,b,c,d}	2.82 ± 0.14 ^{a,b,c,d}

Table 3. Effect of late pregnancy exposure to omega-9 monounsaturated fatty acid on hormonal profile in rats

Values are expressed as mean ± SEM (n=54), ^aP<0.05 vs. Control, ^bP<0.05 vs. D15, ^cP<0.05 vs. D16, ^dP<0.05 vs. D17, ^eP<0.05 vs. D18. One-way ANOVA followed by Tukey-Kramer was used for multiple comparisons test. Day 15= D15, day 16= D16, day 17= D17, day 18= D18, day 19= D19

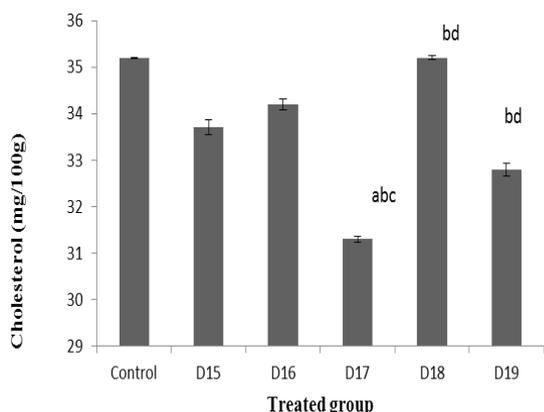


Figure 3. Effect of late pregnancy exposure to omega-9 monounsaturated fatty acid on testicular cholesterol in rats. Values are expressed as mean ± SEM (n=54), ^aP<0.05 vs. control, ^bP<0.05 vs. D15, ^cP<0.05 vs. D16, ^dP<0.05 vs. D17, ^eP<0.05 vs. D18. One-way ANOVA followed by Tukey-Kramer was used for multiple comparisons test. Day 15= D15, day 16= D16, day 17= D17, day 18= D18, day 19= D19

group showed significant positive correlation (r= 0.50, P<0.05) for LDH. The TP level of rats from the control group also showed significant positive correlation (r= 0.50, P<0.05) for LDH.

Testicular cholesterol as a marker of androgen synthesis

There was a significant reduction in the rats from D17 group as compared to the control. Significant reductions also abounded in the testicular cholesterol level of rats from D17 vs. D15, D18 vs. D15, D19 vs. D15, D17 vs. D16, D18 vs. D17, and D19 vs. D17.

Cholesterol level of rats from D19 group demonstrated significant (r=0.95, P<0.05) positive correlation for LDH.

Hormonal profile of rats as a marker of androgen activity

The LH levels of rats from D18 and D19 groups were significantly reduced as compared to the control (Table 3).

The FSH levels of rats from D15, D18, and D19 groups were significantly reduced as compared to the control (Table 3). The FSH level of rats from D17 group demonstrated significant (r = 1.00, P<0.05)

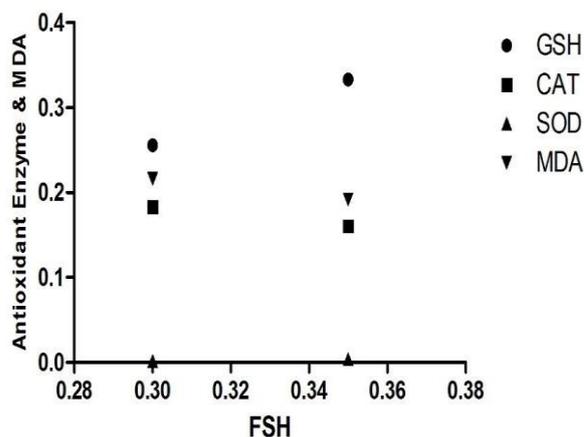


Figure 4. The relationship between follicle-stimulating hormone and the activity of the endogenous antioxidant enzyme system and testicular lipid peroxidation (as measured by malondialdehyde) on day 17, reliability (R) = 1.000. A positive correlation exists between the variables

positive correlation for GSH. Also, FSH level of rats from D19 group demonstrated significant (r = 0.55, P<0.05), (r = 0.54, P<0.05), (r = 0.11, P<0.05) and (r =0.21, P<0.05) positive correlation for GSH, CAT, SOD and MDA, respectively. FSH level of rats from D17 group correlated positively with LDH (r= 1.00, P< 0.05) and FSH level of rats from D19 group also correlated positively with total protein (r=0.67, P<0.05).

The testosterone level was significantly increased in the rats from D17 group as compared to the control, while the testosterone level was significantly decreased in the rats from D18 and D19 treated groups as compared to the control (Table 3). The testosterone level of rats from D17 group demonstrated significant positive correlations of (r=1.00, P<0.05) and (r =1.00, P<0.05) for GSH and CAT, respectively. Also, the testosterone level of rats from D19 group demonstrated significant positive correlations of (r=0.82, P<0.05), (r=0.83, P<0.05), (r=0.99, P<0.05), and (r=0.40, P<0.05) for GSH, CAT, SOD, and MDA, respectively. The testosterone level of rats also from D19 group demonstrated significant positive correlations of (r=0.87, P<0.05) and (r=0.69, P<0.05) for cholesterol and LDH, respectively.

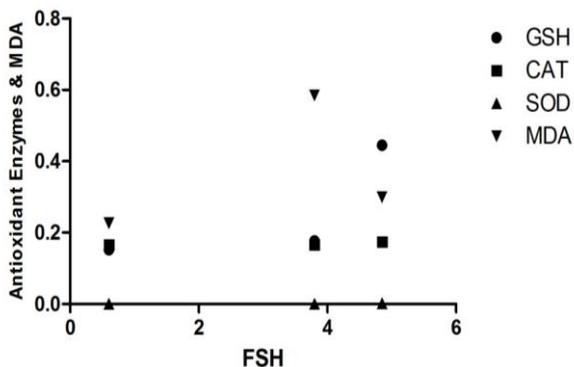


Figure 5. The relationship between follicle-stimulating hormone and the activity of the endogenous antioxidant enzyme system and testicular lipid peroxidation (as measured by malondialdehyde) on day 19, reliability (R) = reliability (R) GSH= 0.553, CAT= 0.541, SOD= 0.116, MDA= 0.2132. A positive correlation exists between the variables

Testicular weight of the rats as a marker for testis activity

The testes weight of the rats from D17, D18, and D19 groups were significantly reduced as compared to the control.

Testicular DNA as a marker for oxidative damage

The fragmentation index of genomic DNA from the testis of rats from D15, D16, D17, D18, and D19 groups were significantly higher as compared to the control. D19 had the highest fragmentation index.

Discussion

The purpose of this study was to evaluate the oxidative status and DNA integrity of male offspring exposed to omega-9 MUFA on different days of late organogenesis.

Sperm count is one of the most sensitive tests for evaluation of spermatogenesis and it is highly correlated with fertility (27). According to Inyang *et al* (28), marked disruption in epididymis malfunction is shown by progressive reduction in sperm motility. Also, in the present study, both sperm count and motility

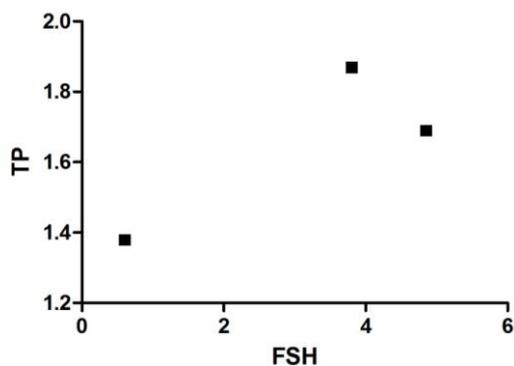


Figure 7. The relationship between follicle-stimulating hormone and total protein on day 19, reliability (R)= 0.670. A positive correlation exists between the two variables

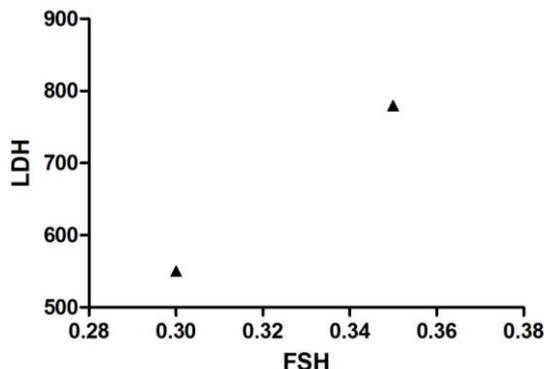


Figure 6. The relationship between follicle-stimulating hormone and lactic dehydrogenase on day 17, reliability (R) = 1.000. A positive correlation exists between the two variables.

were significantly reduced in omega-9 treated rats as compared to the control. The significant reduction in the sperm count and motility levels of rats from treated groups, as compared to the control, may be a result of lipid malondialdehyde (MDA) increase, as its increase has been known to cause spermatogenic disturbances (14). LDH-x is a unique isoenzyme of lactate dehydrogenase, found in the inner mitochondrial membrane of the spermatogenic cells of mature and developing testis, it also plays an important role in transferring hydrogen from the cytoplasm to the mitochondria by redox coupling α -hydroxy acid/ α -keto acid related to spermatozoal metabolism (29); thus, it is referred to as a functional indicator of spermatogenesis and testicular development (30). In the present study, decreased LDH-x level in the rats from D15 and D19 groups may be a consequence of enhanced lipid peroxidation after exposure to omega-9 which may be due to fragmentation of the mitochondrial membrane ultra-structure which in turn affects the membrane bound LDH-x function. Thus, the LDH-x isoenzyme, has been considered as a biomarker of testicular toxicity by some researchers (31-33).

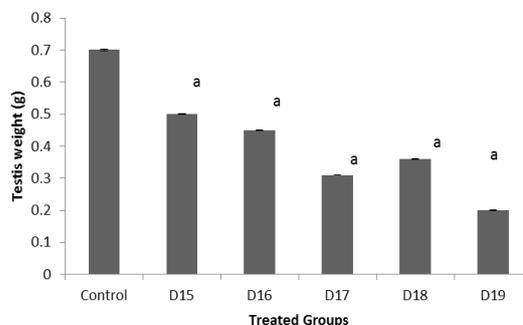
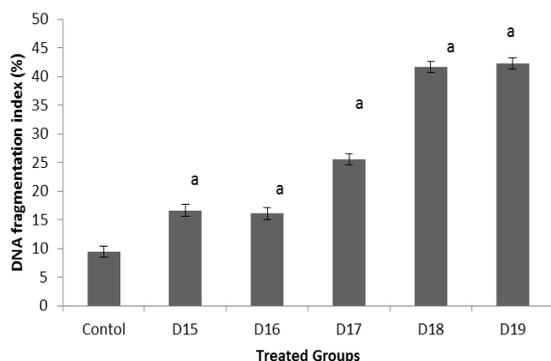


Figure 8. Effect of late pregnancy exposure to omega-9 monounsaturated fatty acid on testis weight in rats. Values are expressed as mean \pm SEM (n=54), ^aP<0.05 vs. control. One-way

ANOVA followed by Tukey-Kramer was used for multiple comparison test. Day 15= D15, day 16= D16, day 17= D17, day 18=



D18, day 19= D19

Figure 9 . Variation in DNA fragmentation index in testes of rats after omega-9 monounsaturated fatty acid exposure in late pregnancy. Values are expressed as mean \pm SEM (n=54), ^aP<0.05 vs. control. (One-way ANOVA followed by Tukey-Kramer was used for multiple comparison tests). Day 15= D15, day 16= D16, day 17= D17, day 18= D18, day 19= D19

There are associations between free radicals or reactive oxygen species (ROS) and oxidative stress (OS) and they play significant roles in reproduction (34). It is suggested that OS is caused by ROS overproduction rather than antioxidant depletion (35). The reactive oxygen species are neutralized by a protective antioxidant defence system in the body comprising of enzymes like catalase (CAT), superoxide dismutase (SOD), and non-enzymes such as glutathione (GSH) and so on (36). These antioxidant enzymes as well as free radical scavengers provide protection against peroxidative damage to the sperm cell and form non-radical end-products (37). In this study, the level of hydrogen peroxide was elevated in the testes of the treated groups, while the testicular superoxide dismutase and glutathione were significantly decreased in rats from treated groups as compared to the control, indicating that oxidative stress was induced by omega-9.

Proteins are known as the most important and abundant macromolecules playing vital roles in the architecture and physiology of the cell as well as cellular metabolism (38). Thus, changes in the protein level suggest that there is a reduction in the synthetic activity in the testes. The protein content of the testicular tissue is a marker of tissue injury, as well as damage (39). In the present study, there was a significant decrease in the testicular protein of the rats from D18 and especially D19 groups as compared to the control as well as between treated groups. This is another indication of oxidative damage induced by omega-9 in the testis. The reduction in testicular protein content can be connected to testicular dysfunction (30). There are also suggestions that testicular protein and steroid hormones are linked as steroid hormones are

sensitive to protein synthesis inhibition (40). This could explain the decrease in testicular protein and cholesterol level as well as the testosterone, FSH, and LH levels observed in the omega-9-exposed rats in this study. The FSH was positively correlated with the testicular protein in D19 group while testosterone was positively correlated with the cholesterol level also in D19 group.

Decreased cholesterol level in D17 and D19 groups is an indication of altered androgen synthesis and its impaired secretion as a result of possible effect of exposure on the size and number of Leydig cells (41). The cholesterol level was most significantly reduced in the testes of the rats from D17 group as compared to the control while there was also significant reduction in the cholesterol level among the treated groups.

The observed decrease in plasma testosterone levels of rats from D18 and D19 groups may be related to the effect of omega-9 localizing between enzymatic steps of testosterone biosynthesis, converting cholesterol to androstenedione, inhibition of 17- α -hydroxylase, cholesterol side chain cleavage as well as 3 β HSD-isomerase steps (34). The most marked toxic effect of omega-9 exposure may also be seen in the Leydig cells. The Leydig cell's function is primarily the secretion of testosterone as well as other steroids. The role of gonadotropins as endocrine regulator of spermatogenesis is well known (42) and LH- induced testosterone secretion by Leydig cell is involved in the regulation of spermatogenesis by targeting androgen receptors in the seminiferous epithelium. FSH also targets receptors within the sertoli cells to regulate spermatogenesis by stimulating the production of numerous sertoli cell factors. Consequently, any factor affecting LH-stimulated Leydig function will in turn interrupt the endocrine regulation of spermatogenesis and affect the reproductive performance of the animal as significant reduction was seen in testosterone, FSH, and LH levels of rats from the treated groups compared to the control. It was observed that FSH levels showed positive correlation with MDA and CAT levels in rats from D19 group as compared to control indicating an association with OS.

The testis is the major site of testosterone production which plays a role in initiation and regulation of spermatogenesis and a reduction in testosterone has the potential to adversely affect normal sexual development in men. Kumar *et al* (43) reported the decreased activity of steroidogenic enzyme and SOD which has been shown to act as an alternate regulatory switch in testicular steroidogenesis and has been linked to the possible role of ROS in reduced steroidogenesis. Activities of SOD and GSH were decreased while level of lipid peroxidation was significantly increased indicating

the occurrence of oxidative stress in the sperm. Oxidative stress, free radical generation and lipid peroxidation have been implicated in the pathogenesis of iatrogenic testicular toxicity (44). Decreased steroidogenic enzyme and SOD in testis as a result of *in utero* exposure to omega-9, could be linked to the possible role of ROS in reduced steroidogenesis. ROS-mediated damage to sperm membrane has been reported to be responsible for impaired sperm motility (45).

The testis is a sensitive indicator of toxicity. The significant decrease in testis weight in D17, D18 and D19 groups as compared to the control, may be due to reduced tubule size spermatogenic and inhibition of steroid biosynthesis of Leydig cells, the site of steroid biosynthesis. Reduced testis weight can cause marked reductions in components of steroidogenic and spermatogenic compartment (46) as seen in this study. Oxidative stress has been implicated in the deterioration of accessory sex organs (47).

Poor quality of sperm DNA appears to be one of the important factors affecting male reproductive ability, both in natural and assisted procreation (12). This has been confirmed by numerous reports in which a higher percentage of spermatozoa with fragmented DNA has been found in infertile men compared to fertile individuals (48-50). Sperm DNA fragmentation can be attributed to physical and environmental factors, infection, inflammation in the male reproductive tract and consumption of sweeteners (51-53). Sperm DNA integrity can be disrupted by three mechanisms namely, defective chromatin packaging, apoptosis and oxidative stress (54, 55). The destructive effect of OS on male gametes is mainly associated with the peroxidative processes of sperm membrane components and DNA fragmentation. This may explain the increase in MDA level, a marker for lipid peroxidation as well as the DNA fragmentation seen in the treated rats. DNA fragmentation increased as the duration of the treatment advanced, consequently, D19 group had the highest DNA fragmentation. An imbalanced seminal plasma antioxidant activity results in the oxidation of various macromolecules such as DNA, proteins and lipids. Therefore, oxidative stress in sperm DNA results in increased DNA fragmentation.

Conclusion

The results of the present study demonstrate that omega-9 MUFA exposure *in utero* decreased sperm motility and sperm count and increased the rate of DNA fragmentation and the level of oxidative stress. These findings on omega-9 MUFA exposure would be beneficial to future infertility research.

Acknowledgement

This research was funded by a grant (CRC/TETFUND/NO.2011/14) from the University of Lagos, Nigeria.

Conflict of Interest

The authors declare no conflicts of interest.

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