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Research Article

Developmental consequences of *in-utero* exposure to omega- 9 monounsaturated fatty acid and its sex-skewing potential in rats

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Keywords:
omega-9, organogenesis, pregnancy, somatic landmarks, foetal development, anogenital index, survival rate.

ABSTRACT

Background: A lot of studies have described the effects of polyunsaturated fatty acids (PUFA) on fecundity and foetal development in experimental animals and in man. There is dearth of information on the effect of *in-utero* exposure to monounsaturated fatty acids on pregnancy outcomes and foetal development in rats. **Methods:** Omega-9 (Oleic acid) was administered by oral gavage at a no-observed-adverse effect level (NOAEL) according to OECD guidelines. Gravid dams (190-220g, n=25) were distributed into five groups of five rats each; $F0_C$: control group was administered 1ml/kg of olive oil, $F0_{EXBM}$ was administered 1000mg/kg of oleic acid for 7days before mating, $F0_{GD1-7}$ was administered 1000mg/kg of oleic acid between gestation day (GD)1-7, $F0_{GD8-15}$ was administered 1000mg/kg of oleic acid between GD8-15, $F0_{GD1-21}$ was administered 1000mg/kg of oleic acid between GD1-21. Gestation length, litter size, offspring anogenital index (AGI) and developmental landmarks were observed. Data were analysed using ANOVA. **Results:** There was a significant increase in gestation length in the $F0_{EXBM}$, $F0_{GD8-15,}$ and $F0_{GD1-21}$ groups compared with the $F0_C$. Litter sizes were significantly reduced in the $F0_{EXBM}$, $F0_{GD1-7,}$ $F0_{GD8-15}$ groups when compared with $F0_C$ ($p< 0.01$). The offspring anogenital index on postnatal day 4 was significantly reduced when compared with control. Mortality was observed in all the three offspring born to the $F0_{GD1-21}$ group. **Conclusion:** Oleic acid has negative effects on pregnancy outcome and foetal development although dams exposed to oleic acid had a shift in offspring sex ratios favouring males.

INTRODUCTION

There are growing concerns about the decrease in male reproductive health and this has been associated with dietary factors (Kumar *et al.*,2009; Sharma *et al.*,2013). The period of pregnancy is a susceptible period by which the foetus is exposed to environmental factors such as diet, maternal stress, smoking, drugs and medications amongst other things that may lead to unsuccessful reproductive functions later in life (Bateson, 2001). Nutrition is an important factor for a successful or an unsuccessful pregnancy state. Under-nutrition of sheep dams during the first two months of pregnancy resulted in changes associated in testicular steroidogenesis. Lumey; (1998), published a study where it was observed that during the Dutch famine birth cohort, there was an increased risk of still birth and prenatal death among offspring of women exposed to famine in the third trimester of pregnancy. A study by Barker, (2000) revealed that women with nutritional deficiencies during pregnancy had small placentas and forty years afterwards, their offspring developed high blood pressure which predisposed them to cardiovascular disease and stroke. Another study reported that male offspring of dams fed a low-protein diet experienced an age-dependent loss of glucose tolerance. They were insulin sensitive and glucose tolerant in early life (Shepherd *et al.*,1997; Petry *et al.*, 2000) but by seventeen months of age they had developed insulin resistance and severe diabetes. Oleic acid is a monounsaturated nonessential fatty acid belonging to the omega-9 (ω-9) family of fatty acids. It can be found in plants, animals and in a large proportion of human dietary intake with a low uptake by the liver and brain. Unlike omega-3 and omega-6

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fatty acids (α- linoleic acid and linoleic acids respectively), omega-9 fatty acids are produced by the body, but are also beneficial when they are obtained in food. If oleic acid is not provided in sufficient quantity, cells cannot produce other important fatty acids, and fatty acid derivatives. Beef and poultry contain 30-45% of oleic acid while oils like grape seed, palm, peanut, soybean and sunflower contain 25-49% oleic acid (Waterman and Lockwood, 2007).

Oleic acid also forms a large percentage of olive oil which is the staple fat of the Mediterranean diet. This oleic acid level is increased in the plasma membrane phospholipids from various tissues in rat and human cells (Escudero *et al.*, 1998; Vicario *et al.*, 1998). Oleic acid is used in the food industry to make synthetic butters and cheeses. It is also used to flavour baked items, candy, ice cream and sodas (Rickman, 2004). Oleic acid has several clinical applications which include its effect on coronary heart disease and its ability to reduce blood pressure, regulation of insulin sensitivity in hepatic steatosis (Win, 2005). It also has beneficial effect on the pathogenesis of vascular diseases whereby it protects low density lipoprotein (LDL) from oxidation (Mata *et al.*, 1997) as well as inducing less monocyte chemotaxis and adhesion on exposure to oxidative stress (Tsimikas *et al.*, 1999). Oleic acid has also shown activity in cancer prevention (Waterman and Lockwood, 2007; Wu *et al.*, 2012). For instance, a study revealed that oleic acid blocks the action of HER-2/neu, a cancer-causing oncogene found in about 30% of breast-cancer patients (Reuters, 2005). Despite its clinical applications, there is dearth of information on the effect of *in-utero* exposure to oleic acid (omega-9 monounsaturated fatty acid) on pregnancy outcomes and foetal development in rats. This study was thus carried out to investigate these indices.

MATERIALS AND METHODS

Animals and Animal Husbandry

Thirty female rats weighing between 190-220g, purchased from the Central Animal House, University of Ibadan, Nigeria were used for this study. Animals were maintained in an airy room under a controlled 12h: 12 h light/dark cycles, relative humidity was 50±5 % at room temperature of 21±1º C. The rats were fed with standard rat chow (obtained from Ladokun feeds, Ibadan, Nigeria) and tap water *ad libitum.*

The study was conducted in accordance with the Use of Laboratory Animals committee of the Central Animal House, University of Ibadan and care was taken to guarantee that animal handlings and experimental protocols conformed with the internationally accepted guideline for laboratory animal use and care (NIH Publication No. 85-23, revised 1985).

Study Design

The rats were mated daily in the ratio of one female to one male. The day of sperm detection in the vaginal smear was determined as day 1 of gestation. Gravid dams were distributed on a random basis into five groups of five rats each as follows:

$F0_{C}$: the control group was administered 1ml/kg of olive oil by oral gavage.

$F0_{EXBM}$ was administered 1000mg/kg of oleic acid by oral gavage for 7days before mating,

$F0_{GD1-7}$ was administered 1000mg/kg of oleic acid by oral gavage between gestation day (GD)1-7,

$F0_{GD8-15}$ was administered 1000mg/kg of oleic acid by oral gavage between GD8-15,

$F0_{GD1-21}$ was administered 1000mg/kg of oleic acid by oral gavage between GD1-21.

Observations

Gravid dams were daily monitored for mortality and signs of general toxicity. Weekly maternal body weight and daily food consumption were recorded during gestation and lactation. The day of parturition as well as litter size, litter birth weight, numbers of live and still born pups on postnatal (PND) 1, and gender ratio were observed. Other variables assessed were gestational mortality and length of gestation in dams, food consumption and body weights in dams and offspring. The litters were kept homogenous thus each dam nursed 9 male offspring (Agnish and Keller, 1997; Almeida and Lemonica, 2007) throughout the lactation period to eliminate the effect of over-nutrition or under-nutrition of some of the offspring. Each group thus had 45 offspring. After 21days, the offspring were weaned to rat chow and tap water. Male offspring were preferred in this study because female offspring are affected by the size of the litter in which they were reared which has been shown to produce long-term effects on their developmental and reproductive competency (Agnish and Keller, 1997) and these effects needed to be avoided in the assessment of the anogenital index.

Developmental Landmarks.

The days required for the appearance of these landmarks were recorded until all offspring in the litters were positive for that developmental variable. The mean day of appearance of each of the above variables were all recorded and compared among groups. The postnatal developmental landmarks are day of 'eye opening'; day of 'pinna detachment'; day of 'fur appearance' and day of 'incisor eruption' (Almeida and Lemonica, 2007; Nunes *et al.*, 2010). All measurements were strictly conducted by researchers blinded to grouping.

Anogenital Index

The AGD measured by scientists blinded to treatment on PND 1 and 4 was used to normalize for potential differences in body weight to calculate the Anogenital Index (AGI). AGI was calculated as distance in mm/cube root of body weight (Christiansen *et al.*, 2009).

Statistics.

Data obtained were expressed as mean ± standard error of mean (mean ± SEM). The significance of the results was evaluated using analysis of variance (ANOVA) and the means were compared using Tukey-Kramer Multiple comparison Test. $P < 0.05$ was regarded as being statistically significant.

RESULTS

Effect of oleic acid on F0

Feed Consumption

There was a significant reduction in the food intake in the three weeks' administration of oleic acid to $F0_{GD1-21}$ group while the $F0_{GD1-7}$ had a significant increase in feed consumption during pregnancy as presented in Fig 1. In Fig 2, there was no significant difference in the feed consumption of dams when compared with the control during the lactation period. Dams in $F0_{GD-1-21}$ group did not wean any offspring because all the offspring died by the end of PND 1.

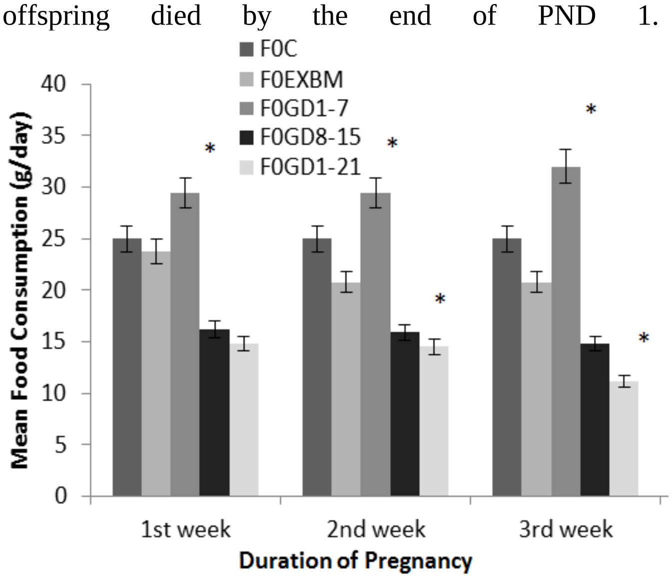

Figure 1: Feed consumption of F0 dams during pregnancy. Values expressed as mean ± S.E.M (n=5) *P<0.05. One-way ANOVA followed by Tukey-Kramer for a multiple comparisons test.

Body Weight

There was no significant difference in the weights of dams during pregnancy (data not shown). The $F0_{EXBM}$ group however, had a significant reduction in their weights on postpartum days 1, 7, 14, 21 (Fig 3).

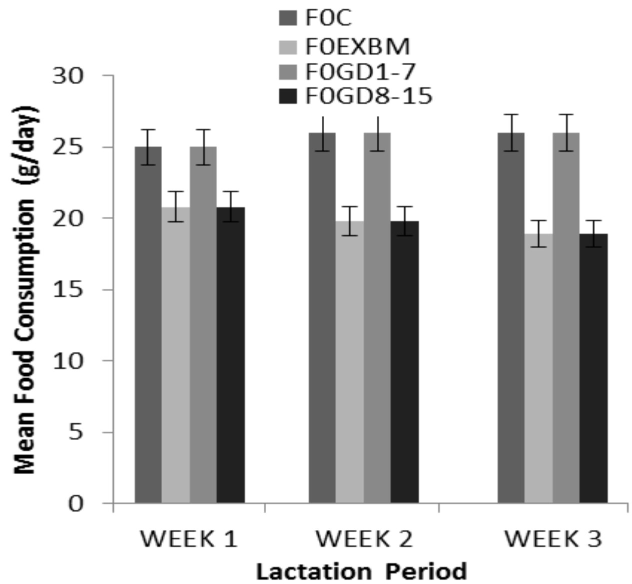

Figure 2: Feed consumption of F0 dams during lactation. Values expressed as mean ± S.E.M (n=5) *P<0.05. One-way ANOVA followed by Tukey-Kramer for a multiple comparisons test.

Gestation and Parturition

There was no gestational mortality recorded in the dams. The gestation length was significantly increased in the $F0_{EXBM}$, $F0_{GD8-15}$, $F0_{GD1-21}$ groups when compared with $F0_C$ group. The litter sizes of the treatment groups were significantly lower than the litter size of the control rats while the litter birth weight was significantly increased in the $F0_{GD8-15}$ group and significantly decreased in the $F0_{EXBM}$, $F0_{GD1-7}$ and $F0_{GD-1-21}$ groups when compared with the $F0_C$ ($P<0.001$). The three foetuses delivered to the $F0_{GD1-21}$ treatment group all died by the end of postnatal day (PND) 1. One of the foetuses that died was observed morphologically to have a headless body.

Effect of Oleic Acid on F1

Feed Consumption

At week 3 there was a significant decrease in the feed intake of the $F1_{EXBM}$, $F1_{GD8-15}$ groups when compared with the $F1_C$ group. By week 4 there was a significant increase in the feed intake of the $F1_{GD1-7}$ group when compared with the $F1_C$ group. A significant decrease was also seen in the trend of the feed intake of the $F1_{EXBM}$, and $F1_{GD8-15}$ groups when compared with the $F1_C$ group at week 5 (Fig 4).

Body Weight

On postnatal days 1 and 4, there was a decrease in weight of $F1_{EXBM}$ and $F1_{GD1-7}$ when compared with $F1_C$. There was an increase in the weight of $F1_{GD8-15}$ when

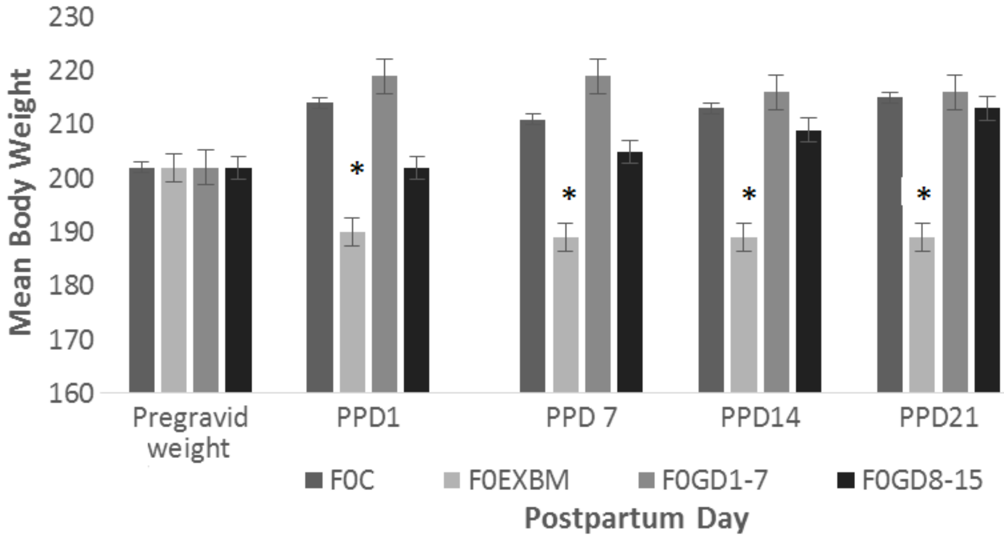

Figure 3: Body weight of F0 dams in different groups during lactation. Values expressed as mean ± S.E.M (n=5) *P<0.05. One-way ANOVA followed by Tukey-Kramer for a multiple comparisons test.

Table 1: Gestational mortality and length in dams; litter size, litter birth weight and survival on postnatal day 1 (PND1) of pups after treatment with oleic acid during pregnancy

Groups	Gestational Mortality (N)	Length of Gestation (days)	Litter size (n)	Litter birth weight (g)	Death of F1 generation at the end of PND 1
$F0_C$	0	21.00 ± 0.20	10.67 ± 0.60	6.03 ± 0.08	NIL
$F0_{EXBM}$	0	27.60 ± 0.24*	5.50 ± 0.88*	5.01 ± 0.07#	NIL
$F0_{GD1-7}$	0	21.40 ± 0.20	7.50 ± 0.76*	5.35 ± 0.10#	NIL
$F0_{GD8-15}$	0	27.60 ± 0.24*	5.50 ± 0.88*	6.59 ± 0.05#	NIL
$F0_{GD1-21}$	0	29.50 ± 0.50*	3.50 ± 0.05*	3.20 ± 0.05#	3

Values are expressed as mean ± SEM (n=5), *P<0.05, #P< 0.001. One-way ANOVA followed by Tukey-Kramer for a multiple comparisons test.

Table 2: Developmental landmarks of F1 offspring after exposure to oleic acid during pregnancy

Groups	Day of Eye Opening	Day of Pinna Detachment	Day of Fur Appearance	Day of Incisor Eruption
$F0_C$	13.78±0.09	18.00±0.00	7.00±0.00	7.00±0.04
$F0_{EXBM}$	17.00±0.00#	21.00±0.00#	9.00±0.05#	9.49±0.08#
$F0_{GD1-7}$	15.49±0.08#	19.69±0.07#	8.00±0.05*	8. 65±0.08#
$F0_{GD8-15}$	17.00±0.00#	21.00±0.00#	9.00±0.05#	9.49±0.08#

Values are expressed as mean ± SEM, n= 45/group (* P<0.05, #P< 0.001). One-way ANOVA followed by Tukey-Kramer for a multiple comparisons test.

compared with $F1_C$ on postnatal day 1. This decrease continued in all groups on PND7. By PND 14, this decrease in weight continued in $F1_{EXBM,}$ and $F1_{GD8-15}$ but an increase was observed in the $F1_{GD1-7}$ group when compared with $F1_{C.}$ Again, on PND 61, the decrease continued in the $F1_{EXBM,}$ and $F1_{GD8-15,}$ groups when compared with the $F1_C$ group while there was an increase in weight of the $F1_{GD1-7}$ when compared with $F1_C$ (Fig 5).

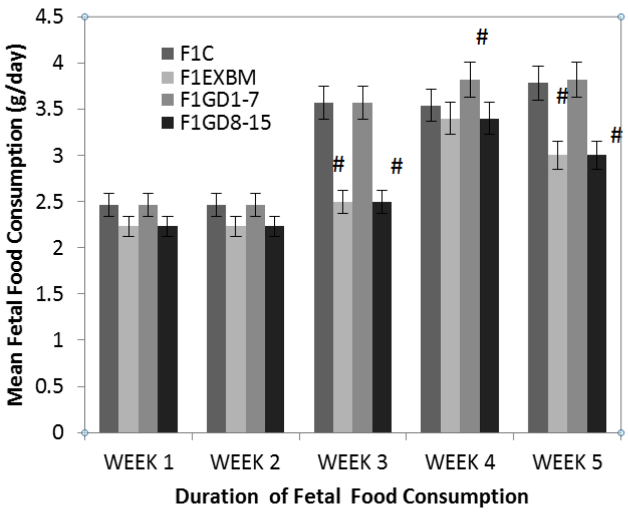

Fig. 4: Feed consumption of F1 offspring after weaning. Values expressed as mean ± S.E.M (n=45) *P<0.05. One-way ANOVA followed by Tukey-Kramer for a multiple comparisons test.

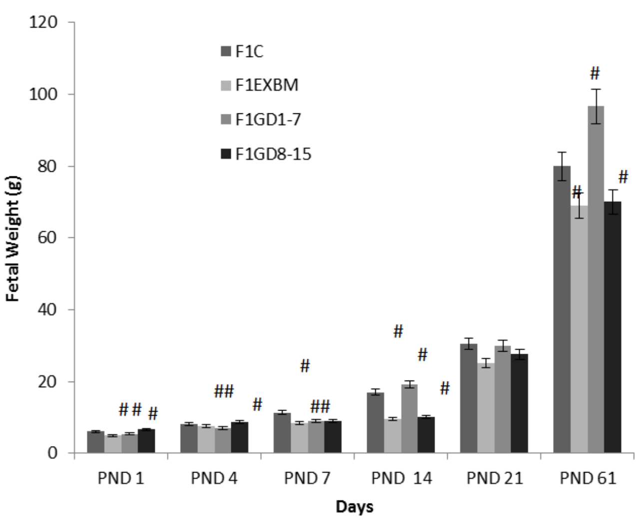

Fig. 5: Postnatal body weight of F1 offspring. Values expressed as mean ± S.E.M (n=45) *P<0.001. One-way ANOVA followed by Tukey-Kramer for a multiple comparisons test.

Anogenital Index (AGI)
The anogenital index (AGI) on postnatal days 1 and 4 were significantly reduced in all offspring when compared with the control (Fig 6).

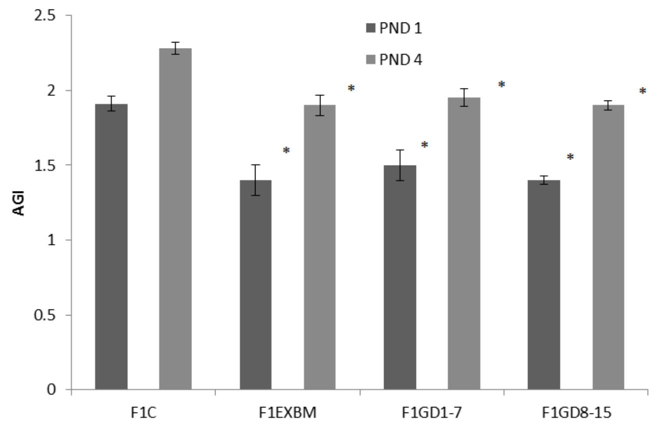

Fig. 6: Anogenital index of F1 offspring on postnatal days 1 and 4. Values expressed as mean ± S.E.M (n=45) *P<0.01. One-way ANOVA followed by Tukey-Kramer for a multiple comparisons test.

Developmental Landmarks
The developmental landmarks were presented in Table 2. The day of eye opening was delayed (p< 0.001) in the offspring of all treated rats when compared with the control. The day of pinna detachment was also delayed (p< 0.001) in the offspring of the treated rats when compared with the offspring of the control rats. The day of appearance of fur was delayed as well in the offspring of all treated rats when compared to the control. The day of incisor eruption was delayed (p< 0.001) in offspring of the treated rats when compared with the offspring of control rats.

DISCUSSION

The effect of *in-utero* exposure to omega-9 monounsaturated fatty acid on pregnancy outcome and foetal development after administration at different stages of pregnancy in rats at the no-observed adverse effect level (NOAEL) was addressed in this study. It was observed in this study that there was no significant reduction in the maternal body weight during pregnancy although there was a reduction in food consumption in the $F0_{GD1-21}$ group after administration of oleic acid in the three weeks of pregnancy. This cannot be classified as maternal toxicity because no clinical signs of toxicity including mortality, morbidity, general appearance and behaviour were observed and there were also no changes in maternal weight. This showed that oleic acid did not have any modulatory effects on the dams during pregnancy. In a study by Lourenço *et al.* (2014), oils from α- linolenic and linoleic food sources did not produce clinical signs of toxicity as well and there were no changes in maternal weights.

Fatty acids have been implicated in gestation, parturition and foetal outcomes in rats. Previous studies have shown that dietary α- linolenic acid increased gestation length and showed differences in the onset of labour (Olsen *et al.,* 1991; Olsen *et al.,* 1992; Baguma-Nibasheka *et al.,* 1999). This study is consistent with the aforementioned report because oleic acid increased gestation length in the $F0_{EXBM}$ $F0_{GD8-15}$, $F0_{GD1-21}$ groups. This increase in gestation length could be an indication of a possible dystocia. It could also be because of the amount of dietary oil used and not necessarily the type of oil used. An instance is the study by Szajewska *et al.,* (2006) that reported an increased maternal dietary intake of food sources from α- linolenic acid in human pregnancy increased gestation length, although it reduced the risk of pregnancy complications (Zhou *et al.,* 2012).

Goetz *et al.* (2007), infers that the term 'survival rate' is used when an offspring lives up to eight days after birth. This statement thus implies that the survival rate of offspring from the $F0_{GD1-21}$ treated group was nil because they all died by the end of postnatal day one. In a study by Yan *et al.* (2013), an increasing ratio of α- linolenic acid to linoleic acid (n-3/n-6) resulted in increased litter size and birth weight. In another report, where linoleic acid was supplemented in the diet of sows, there was an improved embryo survival as well as an increased piglet litter size (Webel *et al.,* 2004; Smits *et al.*, 2011). In this study, the $F0_{GD8-15}$ treatment group was the only group with an increased litter birth weight while other groups had a significant decrease in litter birth weight. In another study, which corroborates with the findings in this study, reduced birth weight was observed in both rats and human exposure to low arachidonic acid (linoleic group) diet (Taylor *et al.*, 2003). In yet another study, pregnant rats administered a diet rich in high linoleic acid resulted in smaller offspring and reduced litter sizes (Amira *et al.*, 2011). The increased litter birth weight observed in the $F0_{GD8-15}$ treatment group could be as a result of increased circulating progesterone concentration in mid-pregnancy while the reduction observed in the other groups could be because high oleic acid (omega-9) in the plasma caused a disturbance in the endometrial lining system which interfered with foetal development.

Several studies have shown that postnatal supplementation with marine oil (n-3 component) resulted in impaired growth which resulted in low birth weight (Amusquivar *et al.,* 2000). Another possibility for a low birth weight in the offspring is that dams on the omega-enriched diet produce either less milk or poorer-quality milk than control females (Fountain *et al.*,2008). Several reports have also indicated that in utero or perinatal exposure to omega-supplemented diets alters the growth of the offspring (Allen and Harris, 2001, Oken *et al.,* 2004; Song *et al.,* 2004; Korotkova *et al.*, 2005).

The closet review on fatty acids in relation to anogenital distance or index was a study by Lourenço *et al.*, (2014). In this study, the female offspring had anogenital distances that did not differ significantly between the different oily vehicles used for di-butyl phthalate (DBP) which contained different ratios of α-linolenic and linoleic (omega 3 and omega 6) fatty acids thus confirming the absence of androgenic effect of the different oily vehicles. As such the shortening in anogenital distance observed in the male offspring was because of the DBP used and not from the oily vehicles. In this study, oleic acid caused a significant reduction in the anogenital index in male offspring an indication of the presence of an anti-androgenic effect.

Delayed postnatal development in offspring of rats fed α- linolenic (omega 3) fish-oil rich diet has been reported (Amusquivar *et al.,* 2000; Herrera, 2002). This delay in developmental landmarks corroborates with our study. This also collaborates with findings that reported that omega-oils alter growth, development and behavioural attitudes in animals.

In the mice, maternal diet enriched with linoleic (omega 6) fatty acids have been shown to shift offspring sex ratio to favour females while diet enriched with α- linolenic (omega 3) had no effect on sex ratio (Fountain *et al.*,2008). In this study, it is interesting to note that there were more male-biased litters than females. Previous studies in opossums and humans suggested that females on a diet supplemented with PUFA gave birth to more sons than daughters (Austad and Sanquist, 1986, Crawford *et al.*, 1987). This study agrees with an earlier suggestion that female mice on a very high-fat diet comprised largely of lard produced more male offspring because of the high content of palmitic, stearic, and oleic acids in lard (Fountain *et al.*,2008). The skewing of more male offspring in this study could be because of the oleic acid exposure, or because of the dose of the exposure of the dams to the oleic acid or because of the type of animal species used. The fat composition of diets, especially the fatty acids, could influence several events in the reproductive process. Some of these processes are oocyte maturation, the timing of ovulation (Bilby *et al.,* 2006), production of chemo attractants by the oocyte (Kubagawa *et al.,* 2006), prostaglandin synthesis (Henderson *et al.,* 1989), and properties of the reproductive tract (Fountain *et al.*,2008), which might, in turn, adjust the relative fertilization abilities of X and Y chromosomes or provide an advantage to conceptuses of one sex over the other during their development (Fountain *et al.*,2008).

The importance of this study is that exposure of dams to oleic acid had a negative effect on pregnancy outcomes, while the offspring of such dams had delays in attaining their developmental landmarks as well as having reduced anogenital index in the males. The dams exposed to oleic acid however, had a shift in offspring sex ratios favouring males.

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