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Antiandrogenic and estrogenic characteristics of oleic acid: Experimental design incorporating endocrinal, testicular, and sperm analysis

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ABSTRACT

This research was to investigate whether *in utero* exposure to oleic acid (OA) could modify the antiandrogenic and estrogenic endocrine functions of the testis during puberty. Pregnant rats were grouped into four groups, with five rats in each group, as follows: control was given 1 mL/kg of olive oil; pretreatment was given 1000 mg/kg OA for seven days before mating; D7 was given 1000 mg/kg OA at gestation day (GD)1–7; and D14 was given 1000 mg/kg of OA at GD8–14. The male offspring delivered were studied into puberty. Hormone levels, age of puberty, and oxidative parameters were determined. The estrogenic properties of oleic acid observed in this study included decreased serum testosterone and a reduction in the epididymis, prostate, and testis weights. Decreased sperm motility and viability, decreased testosterone synthesis, reduced weight of androgen-dependent organs, and delayed onset of puberty were reported as anti-androgenic properties. Testicular MDA levels were significantly higher in OA-exposed rats, compared to control rats. In conclusion, although OA possesses both estrogenic and antiandrogenic properties, the estrogenic characteristics were less pronounced. The antiandrogenic characteristics, steroid hormone inhibition, decrease in sperm variables, and increase in oxidative stress were more distinct.

Keywords: oleic acid; estrogenic; antiandrogenic; oxidative stress; endocrine-disrupting chemicals

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1. INTRODUCTION

Investigation into endocrine-disrupting chemicals (EDCs) must continue with an intensified knowledge of their effects on human health because of their ever-increasing numbers. The United States Environmental Protection Agency defines an endocrine-disrupting chemical as 'an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes (Kavlock et al., 1996). EDCs have anti-androgenic and/or estrogen-like characteristics. They range from persistent pesticides and herbicides to per- and poly-fluoroalkyl substances (PFAs) found in non-stick pots and pans, food packaging items, and the lining of aluminum cans, methoxychlor, biocides, heat stabilizers, plastic contaminants, chemical catalysts, drinking water (Sunderland et al., 2019), and even dietary components. Phytoestrogens are considered as EDCs (Bar-El and Reifen, 2010). Plant-derived oils like lavender and tea tree oils are also called EDCs (Henley and Korach, 2010).

There are suggestions that EDCs are a reason behind the reproductive health deterioration of males (Sidorkiewicz et al., 2017). The WHO modified its recommended semen values to lower rates. There are suggestions that this could be because of the decrepitude in sperm quality in men worldwide (Cooper et al., 2010). Primary testicular dysfunction is the most common male reproductive dysfunction known. It is identified in 75% of male patients, who are infertile (Sidorkiewicz et al., 2017). The etiology of 50% of decreased sperm count and sperm quality cases is not known (Feki et al., 2009). Research is pointing to environmental exposures to low doses of EDCs in fetal and adult repro-endocrine systems as probably the reasons behind the baffling male infertility experienced worldwide (Marques-Pinto and Carvalho, 2013). There is therefore a need to understand the EDCs in dietary substances and their potential effects on male reproductive well-being, which might enable the prevention of the effects of EDCs on male infertility and their likely long-term sequelae.

Oleic acid (OA) is a major fatty acid in the monounsaturated group. OA is found in animals, plants, and mostly in human diets and has low uptake via the brain and liver (Piccinin et al., 2019). Although made in the body, oleic acid is also useful when obtained through food (Piccinin et al., 2019). An inadequate supply of OA can lead to cells not producing other vital fatty acid offshoots. OA is also found in poultry, beef, and oil seed plants, all of which are consumed by humans, including men (Smith and Smith, 2016). OA profusely exists in the fat tissues of meaty components of animals and oil seeds, as well as plant fats (Sindhu and Singh, 2022). It is the most abundant fatty acid found in olive oil. OA has been reported to have beneficial effects (Arruzazabala et al., 2011; Oyelowo et al., 2019) as well as detrimental effects (Oyelowo and Adegoke, 2016; Oyelowo and Bolarinwa, 2020) on the male reproductive system. Most cases (50%) of infertility, according to research, are caused by male factors. Non-genetic sources like diets have been linked to male infertility. Research on nutrition and male reproduction is currently focusing on dietary fatty acids, which may have either beneficial or detrimental effects (Esmaeili et al., 2015). This study was thus carried out to determine the androgenic and estrogenic properties of OA, considering rising interest in dietary fatty acids and male reproduction. This was experimentally investigated in pubertal male rats exposed to OA *in utero.*

2. MATERIALS AND METHODS

2.1 Materials

Olive oil used in this study was imported from Andalusia Spain and was purchased in a local store while OA was purchased from Merck in Germany. The serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone levels were determined via the enzyme-linked immunoassay (ELISA), according to the manufacturer's instructions. The ELISA kits were manufactured by Monobind Inc., California (USA), with batch number EIA-6K2A2. All other chemicals and reagents used were of analytical grades.

2.2 Experimental animals

Twenty pro-oestrus female rats that weighed between 190 and 220 g were used for the study. They were bought from the Central Animal House of the Institution and maintained in an airy room under a controlled 12 h:12 h light/dark cycle; relative humidity was 50±5% at a room temperature of 21±1°C. The dams were fed with standard rat chow and tap water *ad libitum.* The rats were mated daily in the ratio of one female to one male. The day of detection of sperm from the smear of the vagina was established as Gestation Day 1. Gravid dams were distributed randomly into four groups of five rats each: control was administered 1 mL/kg of OA; pretreatment was administered 1000 mg/kg of OA for 7 days prior to mating; D7 was administered 1000 mg/kg of OA between gestation days (GD)1–7; and D14 was administered 1000 mg/kg of OA between GD8–14. All administrations were carried out by oral gavage. Dams delivered their offspring naturally. Each dam in each group was allowed 8 pups to nurse throughout the lactation period. The reason was to eliminate the effect of overnutrition or undernutrition that may occur in some pups if there are too many. This research was conducted according to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and was approved by the University's Ethical Review Committee.

2.3 Determination of the age of puberty

Preputial separation, meant to ascertain the age of puberty, was assessed in male offspring from post-natal day (PND) 40 until complete preputial separation was achieved. The mean age of preputial separation is between 39 and 45 days, prior to the emergence of mature sperm in the caput epididymis (Loeffler and Peterson, 1999). Pups were scrutinized daily between 9:00 a.m. and noon for the age of preputial separation. Body and organ weights were recorded when the preputial separation took place.

2.4 Sample preparation

At puberty, the rats were intraperitoneally given pentobarbital sodium (50 mg/kg) to make them unconscious. A heart puncture was used to obtain blood samples, which were then placed in plain sample bottles and left to stand for 30 min before being centrifuged for 5 min at 3000 rpm. The serum was frozen until the time for the biochemical assay analysis. The epididymis, testes, seminal vesicles, and prostates were instantly removed and cleared of any connective tissues that might have been stuck, blotted, and weighed.

2.5 Hormonal analysis

At puberty, hormonal analysis was carried out. Dissections were done between 8:00 a.m. and 10:00 a.m. to reduce circadian effects on testosterone and other hormones (Meerts et al., 2004). The serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone levels were determined via the ELISA, according to the manufacturer's instructions.

2.6 Sperm analysis

The sperm count, sperm motility, and sperm morphology were analyzed as illustrated in earlier research (Adekunbi et al., 2016).

2.7 Measurement of malondialdehyde testicular level and testicular antioxidants

The malondialdehyde (MDA) level in the testicular homogenate was measured by Uchiyama and Mihara's method (1978). It involves thiobarbituric acid reactive substances (TBARS) production. A pink complex is created, having an absorption of 535, which is chosen as an index of lipid peroxidation. Superoxide dismutase (SOD) enzyme activity in the testicular homogenate was analyzed by the method of Sun and Zigman (1978). The reaction occurred in 0.05 M sodium carbonate buffer, pH 10.3, and was initiated by adding epinephrine in 0.005 N HCl. GSH was determined based on Ellman's reagent 5, 5' dithiobis-2-nitrobenzoic acid (DNTB) with the thiol group of the GSH reaction at pH 8.0 to produce 5-thiol-2-nitrobenzoate. The reaction is yellow in color and has a wavelength of 412 nm. Agilent UV-visible spectrophotometer was used in all assays. The reduced glutathione (GSH) content of the testicular homogenate was determined by the method of van Dooran et al. (1978).

2.8 Statistical analysis

Data were expressed as mean±standard error of the mean (SEM). The statistical analysis was evaluated using analysis of variance (ANOVA), and the means were compared using the Tukey-Kramer comparison test. The $p<0.05$ level was regarded as being statistically significant.

3. RESULTS

3.1 Hormonal levels

Testosterone levels significantly decreased ($p<0.05$) in all offspring exposed to OA *in utero*, compared to the offspring of the control rats. FSH level was significantly decreased ($p<0.05$) in all offspring exposed to OA *in utero*, compared to the control. The LH level was also decreased in all offspring exposed to oleic acid *in utero*, compared to the offspring of the control rats (Figures 1 to 3).

3.2 Onset of puberty in rats

Pubertal age was significantly delayed in all offspring exposed to OA *in utero*, compared with the offspring of control rats (Figure 4).

3.3 Androgen organ weights

The epididymal weight was significantly increased in the offspring of the D7 group, compared to the control. The value of the epididymal weight of offspring from the pretreatment group was significantly decreased, when compared to the offspring of the control rats. The testis weight was significantly decreased in the offspring of pretreatment and D14 OA-exposed rats, when compared to the offspring of the control rats. The seminal vesicle weight was significantly increased in the offspring of the D14 OA-exposed rats, compared to the offspring of control rats. The prostate weight was significantly increased in the offspring of the D7 OA-exposed rats, compared to the offspring of control rats (Table 1).

3.4 Sperm analysis in rats

The level of sperm motility was significantly decreased in the offspring of OA-exposed rats, when compared to the offspring of the control rats. The level of sperm viability was significantly decreased also, in the offspring of OA-exposed rats. The abnormal sperms were significantly increased in the offspring of OA-exposed rats (Table 2).

3.5 Malondialdehyde testicular level and testicular antioxidants rats

The testicular superoxide dismutase level in the offspring of oleic acid-exposed rats was significantly reduced, when compared to the offspring of control rats. Testicular GSH levels in the offspring of the pretreatment and D14 groups were significantly reduced, when compared to the offspring of control rats, while the GSH level in the offspring of the D7 group was significantly increased. Malondialdehyde levels significantly increased in all groups of offspring exposed to OA *in utero*, compared to the offspring of control rats (Table 3).

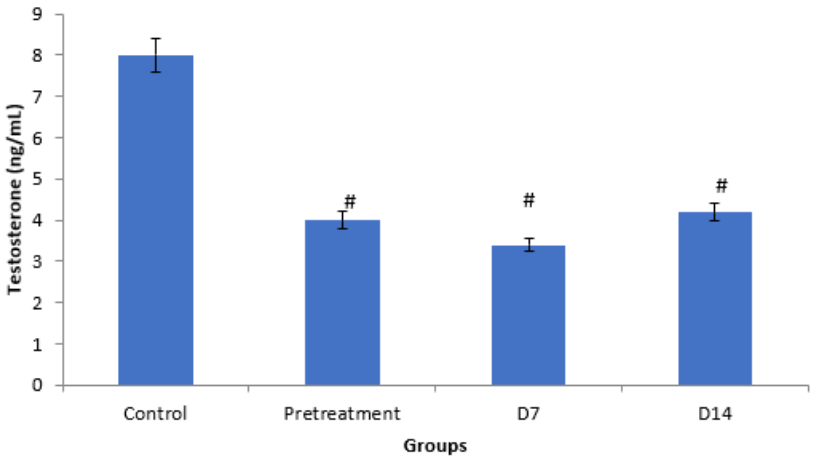

Figure 1. Testosterone levels in all offspring exposed to oleic acid *in utero*, compared with the offspring of control rats
Note: Values are expressed as mean±SEM of 8 rats per group, #$p<0.05$ VS. control.

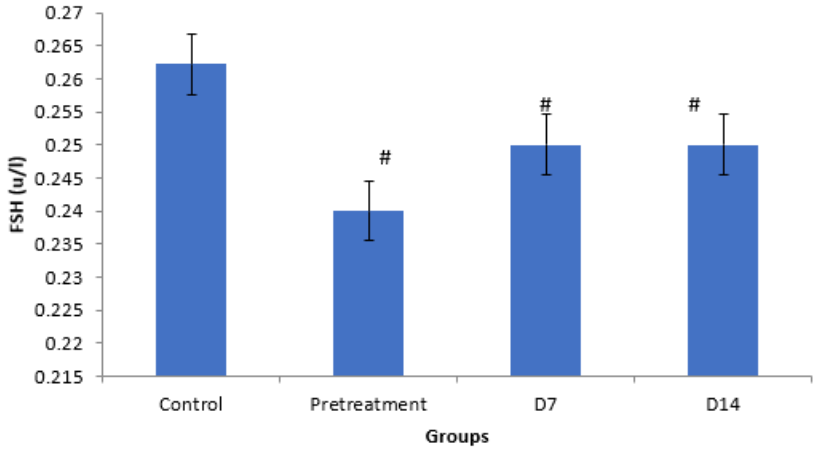

Figure 2. FSH levels in all offspring exposed to oleic acid *in utero*, compared with the offspring of control rats
Note: Values are expressed as mean±SEM of 8 rats per group, #p<0.05 VS. control. FSH**:** follicle-stimulating hormone.

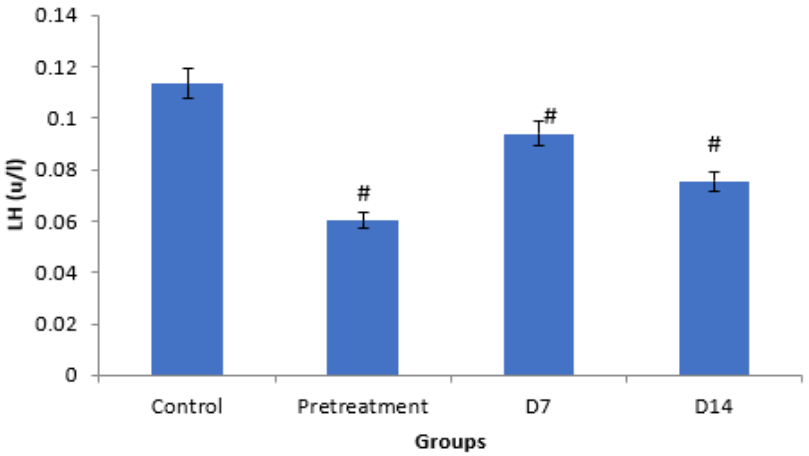

Figure 3. LH levels in all offspring exposed to oleic acid *in utero*, compared with the offspring of control rats
Note: Values are expressed as mean±SEM of 8 rats per group, #p<0.05 VS. control. LH: luteinizing hormone.

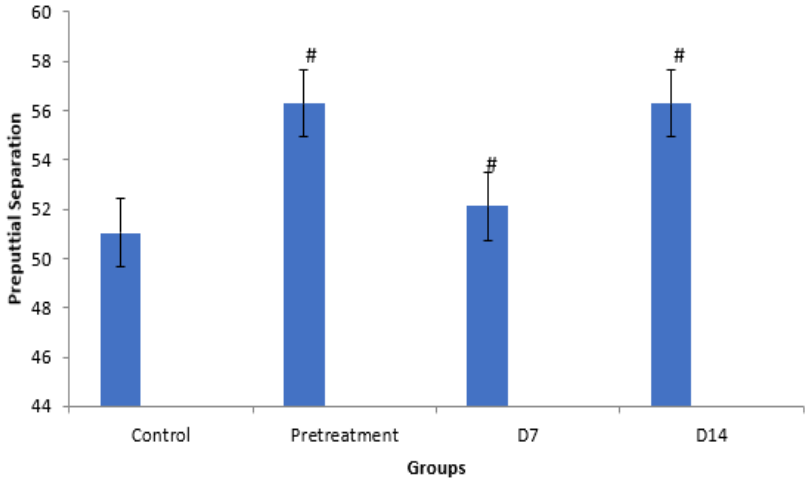

Figure 4. The onset of puberty (preputial separation) in the offspring
Note: Values are expressed as the mean±SEM of 8 rats per group, #p<0.05 VS. control.

Table 1. Androgenic organ weights of offspring exposed to oleic acid *in utero*, compared with the offspring of control rats

Groups	Epidydimis (g)	Testis (g)	Seminal vesicle (g)	Prostate (g)
Control	0.03±0.00	0.69±0.00	0.06±0.00	0.03±0.00
Pretreatment	0.02±0.01*	0.50±0.00*	0.05±0.00	0.03±0.00
D7	0.05±0.01*	0.70±0.00	0.05±0.00	0.05±0.00*
D14	0.04±0.00	0.53±0.01*	0.07±0.00*	0.03±0.00

Note: Values are expressed as mean±SEM, n=8. *p<0.05 was significant when compared with the control.

Table 2. Epididymal sperm characteristics of offspring exposed to oleic acid *in utero*, compared with the offspring of control rats

Groups	Sperm motility (%)	Sperm viability (%)	Abnormal sperm (%)
Control	87.00±3.13	87.69±4.75	3.10±0.01
Pretreatment	50.00±2.50*	80.86±5.39*	10.80±3.25*
D7	59.00±2.23*	78.85±5.34*	6.90±2.10*
D14	40.00±4.15*	76.22±5.41*	7.90±1.70*

Note: Values are expressed as mean±SEM, n=8. *p<0.05 was significant compared with the control.

Table 3. Malondialdehyde testicular level and testicular antioxidants of offspring exposed to oleic acid *in utero*, compared with the offspring of control rats

Groups	MDA (nmol/mL)	SOD (µmol/mL)	GSH (µmol/mL)
Control	1.00±0.09	7.20±0.45	1.84±0.04
Pretreatment	4.46±0.07*	2.20±0.05*	0.5 ±0.00*
D7	4.50±0.05*	2.79±0.00*	2.29±0.31*
D14	5.10±0.08*	2.40±0.05*	0.60±0.00*

Note: Values are expressed as mean±SEM, n=8. *p<0.05 was significant, compared with the control. MDA: malondialdehyde; SOD: superoxide dismutase; GSH: glutathione

4. DISCUSSION

The estrogenic properties of OA observed in this study included decreased serum testosterone, a reduction in epididymis and prostate weights, and a reduction in testicular weight. This is like a previous report by Yamamoto et al. (2003), who demonstrated the effects of diethylstilbesterol and estrogenic characteristics on the reproductive system in the rat offspring. Although OA showed less estrogenic properties *in utero,* exposure to OA affected testicular development and related processes, as reported by Sidorkiewicz et al. (2017). The authors expatiated on the estrogenic and antiandrogenic properties of EDCs and how they affect the male reproductive system. OA, just like other endocrine-disrupting chemicals (EDCs), exerts antagonist or agonist effects in a tissue-detailed way. In a previous study, male animals were exposed to OA at puberty, as opposed to this study, where OA was exposed *in utero* to male rats. In that study, there was a significant increase in LH and FSH levels with no significant difference in testosterone levels. A significant increase in the serum oestradiol levels indicated an estrogenic property of OA (Aisoni et al., 2022). Estrogenic EDCs can also put forth more general effects via the stimulation of oxidative stress (Knez, 2013).

The reduced weight of the testis and other androgen-dependent organs, which will result in lower sensitivity to androgens, are examples of anti-androgenic properties. Decreased sperm motility and viability, decreased testosterone synthesis, malformation of the epididymis, abnormal sperms, and delayed onset of puberty reported in this study are all anti-androgenic properties exhibited by OA exposed *in utero* to male rats. These anti-androgenic properties have been reported by researchers who studied substances possessing estrogenic and anti-androgenic properties (Higuchi et al., 2003; Axelstad et al., 2018). Anti-androgenic compounds act through different mechanisms, like steroid synthesis or androgen receptor antagonism impediment. Anti-androgenic compounds interrupt the androgen-signaling pathway while androgen-sensitive tissues are impacted (Blystone et al., 2009; Orton et al., 2014). The steroidogenic enzymes in the steroid hormone biogenesis pathway are significant targets for EDC actions. Steroid hormones regulate several developmental and physiological processes from fetal life to adulthood (Svechnikov et al., 2010). Steroid hormones have cholesterol as their precursor. Testosterone biosynthesis takes place in the Leydig cells and is prompted by the LH (Tremblay, 2015). LH binding to its receptor in Leydig cells involves an increase in intracellular cyclic adenosine monophosphate, translocation of cholesterol into mitochondria, conversion of cholesterol to pregnenolone catalyzed by the cholesterol side-chain cleavage enzyme (P450scc), and other stages until the conversion of androstenedione to testosterone catalyzed by 17 β-HSD in the smooth ER microsomes of the testis (Enangue Njembele et al., 2014; Tremblay, 2015). In another study investigating the outcome of *in utero* exposure to OA on stress and reproductive hormones in male rats, the result reported reduced testosterone, FSH, and LH levels (Oyelowo and Bolarinwa, 2020). The decrease in the anogenital distance (AGD), an anti-androgenic characteristic (Foster, 2006), has been reported in *in utero*

exposure to OA in a previous study (Oyelowo and Bolarinwa, 2017).

In this study, the *in utero* exposure to OA terminated on gestation day 14. In a previous study, *in utero* exposure to OA was on gestation day 15–19. Although there was a significant increase in FSH and LH levels on days 18 and 19, testosterone levels decreased. Other androgen markers like cholesterol and protein levels were significantly decreased, when compared to the control. The sperm motility and count were also decreased. The testicular fragmentation index of DNA was significantly higher, compared to the control, and the male offspring from gestation day 19 had the highest fragmentation index (Oyelowo and Adegoke, 2016). Indirect action of EDCs via the induction of epigenetic mechanisms like an indication of DNA hypomethylation has been reported (Doshi et al., 2011; Singh and Li, 2012). Anti-androgenic activity has been reported in fatty acids (Liu et al., 2009). Anti-androgens compete with the peripheral androgen receptors; consequently, they impede the effect of endogenous or exogenous androgens. Inhibiting 5α-reductase is believed to be a mechanism by which anti-androgens carry out their activities (Akamine et al., 2009). Although anti-androgens impede androgen actions, there are indications that any remedy that could reduce androgenic hormone action is believed to possess enormous possibilities in tackling benign prostate hyperplasia (BPH) or prostate cancer. Oleic acid found in saw palmetto lipid (Arruzazabala et al., 2011) and pomegranate, olive, blackseed, shea butter vegetable, groundnut, sardine, and repeatedly heated palm oils (Oyelowo et al., 2019) have been reported to address experimental BPH. The reduction in male hormones after *in utero* exposure to OA has been reported elsewhere (Oyelowo and Bolarinwa, 2020).

When the testes are exposed to EDCs, diverse dysfunctions of spermatogenesis could occur, depending on the developmental stage at which the exposure occurred, which could range from fetal life to adolescence or adulthood. In this study, OA was exposed to male rats *in utero.* This is like a study by Anway et al. (2006), which reported that vinclozolin had antiandrogenic properties (Kelce et al., 1998), when it was *in utero* for the male, which resulted in sperm disruption. An *in vivo* administration of bisphenol A at 200 mg/kg body weight per day resulted in significantly reduced sperm parameters in male mice due to disturbances in meiotic progression, especially through the first wave of spermatogenesis and apoptosis in testicular cells (Zhang et al., 2013; Xie et al., 2016). When testicular weight decreased, as reported in this study, it was because of the decrease in spermatogenic elements and spermatozoa (Aly and Azhar, 2013; Aly et al., 2016). The process of spermatogenesis is a very coordinated and vigorous procedure, involving division and differentiation stages. When cell proliferation, differentiation, and apoptosis are balanced, normal spermatogenesis occurs, and impaired testicular function would occur if any alteration to its regulation ensued (Giampietri et al., 2005), as reported in this study.

EDCs disrupt the prooxidant/antioxidant balance of cells and induce oxidative stress via the generation of reactive oxygen species (ROS) like superoxide anion and hydrogen peroxide. Although reactive oxygen species have been shown to play a vital role in the defense mechanisms against pathological conditions, excessive generation of free oxygen radicals may damage tissues (Puppel et al., 2015). The excessive generation of reactive oxygen species has been shown to cause peroxidative damage to the plasma membrane, which leads to impaired sperm function (Kefer et al., 2009), which is similar to this study.

The sperms are especially vulnerable to the damaging effects of ROS because their cell membrane contains large amounts of unsaturated fatty acids, which can be oxidized due to lipid peroxidation, and the cytoplasm has only a low concentration of the enzymes that can neutralize ROS (Walczak-Jedrzejowska et al., 2013). Lipid oxidation results in a loss of membrane integrity and an increase in its permeability, inactivation of cellular enzymes, structural DNA damage, cell apoptosis, etc. This results in decreased sperm count and activity, decreased motility, and abnormal morphology (Sidorkiewicz et al., 2017). In that study, increased testicular MDA levels, a marker of lipid peroxidation, reduced testicular antioxidant activities, decreased sperm motility, and abnormal sperm morphology were reported. The generation of ROS by reducing the activities of antioxidant enzymes while increasing lipid peroxidation results in oxidative stress in the epididymal sperm of rats (Bindhumol et al., 2003) and the testis (Kabuto et al., 2004).

5. CONCLUSION

OA could impede endogenous ligand binding to the estrogen and androgen receptors. OA exerted its anti-androgenic action by directly inhibiting testosterone synthesis in the Leydig cells as well as inhibiting steroidogenic enzymes, leading to the reduction of hormones. The increase in oxidative stress level is also one of the mechanisms associated with oleic acid acting as an anti-androgenic compound on male reproduction by reducing the levels of the sperm parameters, increasing abnormal sperms, inducing reactive oxygen species, lipid peroxidation, and apoptosis of spermatocytes. These are possibly the mechanisms by which OA exhibits its estrogenic and anti-androgenic properties in *in utero* exposures in male pubertal rats.

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