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Influence of Oleic Acid Supplementation on Reproductive and Immune Parameters in Diet-Unrestricted Female Wistar Rats

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ABSTRACT

This study aimed to evaluate the immune-reproductive and endocrine effects of exogenous oleic acid in mature female Wistar rats on an unrestricted diet. Eighteen mature Wistar rats with normal oestrous cycles were randomly divided into three groups: a control group and two experimental groups (low-dose and high-dose), each consisting of six rats. For three weeks, the rats in the experimental groups received daily oral doses of oleic acid at either 500 mg/kg or 1000 mg/kg of body weight. In contrast, the control group received 0.5 ml of distilled water. After the treatment, the oestrous cyclicity, serum hormone levels, reproductive organ weights, and blood parameters were analysed through a full blood count. The results showed no significant changes ($p > 0.05$) in oestrous cyclicity, serum oestrogen, progesterone, or luteinizing hormone levels. However, there was a significant increase ($p < 0.05$) in the weight of the relative reproductive tract, as well as in the number of mid-cells and neutrophils. Conversely, there was a substantial decrease ($p < 0.05$) in the relative lymphocyte count. Overall, the research indicated that oleic acid does not influence oestrous cyclicity or levels of serum oestrogen, progesterone, and luteinizing hormone in rats on an unrestricted diet. However, it does appear to have immunoregulatory effects on inflammatory cells.

Key words: Female reproductive organ; Immune response; Oleic acid; Steroid hormones; Unrestricted diet; Wistar rats.

INTRODUCTION

It is widely acknowledged that fatty acids differ significantly in their metabolic and physiological properties. Many foods, including meat, nuts, and olive oil, contain monosaturated omega-9 fatty acids, such as oleic acid. It is generally accepted to be a beneficial non-esterified fatty acid. Research on oleic acid has demonstrated that it can affect reproduction in either a favorable or detrimental way; In farm animals, dietary components like fatty acids, are used in enriching lactating cows in the dairy industry to improve their lactating ability and milk quality (Burch *et al*., 2020; Prom *et al*., 2021). However, endogenous oleic acid is found at high levels in the blood of livestock that have negative energy balance and a decline in their fertility (Churakov *et al*., 2021; Zhou *et al*., 2022). In women on in-vitro fertility treatment, there was a direct relationship between the consumption of oleic acids and an increased infertility rate (Jahangirifar *et al*., 2021). Herrera-Camacho *et al.* (2011) made a comprehensive review of the positive and negative effects of lipids and polyunsaturated fatty acids on steroidogenesis and female fertility but, concluded that the type of fatty acid in the lipidic diet is responsible for the varying results of researchers. Oleic acid acts as a biomolecule and participates in the metabolic and structural function of an organism. It is reported that in vitro, oleic acid interferes with the regulation of genes involved in steroidogenesis and with progesterone and 17 beta oestradiol production (Yenuganti *et al*., 2016). Oleic acid has been reported to significantly reduce testosterone levels by inhibiting cholesteryl esterase activity in mouse Leydig cells and decreasing cellular cholesterol content (Meikle *et al*., 1996). In vitro, it induces selective lipid accumulation and alters the lipid type stored in ovarian granulosa cells, thereby affecting steroidogenesis (Zhou *et al*., 2022).

Oleic acid is also reported to affect female reproduction by regulating the release or production of immune cells that participate in female fertility. A diet high in monounsaturated fats (MUFAs) has been linked in several studies to effects on immunomodulatory processes (Taha-Abdulaziz et al., 2019; Santa-María*et al*., 2023). Verlengia *et al.* (2002) showed that mineral, a synthetic oleic acid inhibits Jurkat T cell growth in vitro and lowers the generation of IL-2 and INF-gamma. Cell culture and in vivo studies indicate that different fatty acid-type diets can influence lymphocyte proliferation, lymphocyte production of cytokines, and natural killer cell activity in different degrees or with contradicting results (Calder *et al*., 2002).

Given this context, the current study aimed to ascertain the relationship between some female reproductive endocrine indices and immunoregulatory cells affected by exogenous oleic acid in the diet-unrestricted normal

cycling female Wistar rat model. We hypothesise that exogenous oleic acid affects the reproductive endocrine and immune systems of diet-unrestricted Wistar rats.

MATERIALS AND METHODS

Experimental Animals

Normal cycling virgin Wistar rats (n= 18, 10 – 12 weeks old, 151± 24g) were used for this experiment. They were housed in standard metallic cages in a 12:12 hours light: dark cycle environment. The rats were fed Animal Care® standard growers feed comprised of Crude protein (16.5.5 %), Fat (6.00 %), Crude fibre (6.00 %), Calcium (3.00 %), Phosphorus (0.45 %), Metabolizable energy (2650 kcal/kg) and provided water *ad libitum*. The rats were allowed to adapt to the environment for two weeks before the commencement of the research work.

Experimental Design

The 'Guide to the Care and Use of Experimental Animals' NRC (2011) was followed in the general care and use of the animals. Vaginal cytology was done to identify and select eighteen rats that had a regular 4–5-day oestrous cycle for three cycles. The eighteen rats were weighed, and sub-divided into three groups, having six rats per group, and treatment began when the rats were on dioestrus. The first group (Control group) was administered 0.5 ml distilled water, while the second (Low-dose) and third (High-dose) groups were treated with Oleic acid (OA) (364525-IL Sigma-Aldrich, USA) at a dosage of 500 and 1000 mg/kg body weight, respectively. The selected doses for this study were deliberately set below the No Observable Adverse Effect Level (NOAEL) as outlined by the Organization for Economic Cooperation and Development (OECD) guidelines. This choice was informed by prior research from Oyelowo and Bolarinwa (2017), which found a notable shift towards male foetal gender in pregnant Wistar rats given 1000 mg/kg body weight of oleic acid (OA) orally. A lower dose of 500 mg/kg body weight was included to effectively assess dose-response relationships. The treatment was administered daily for 15 days *per os* with an oral canular, and vaginal smears were collected daily for vaginal cytology. At the end of the treatment, the rats were weighed. The rats were lightly sedated with ketamine before blood samples were collected from the median cantus of the eyes into EDTA vacutainer tubes for haematology, and plain vacutainer tubes for hormone analysis. The rats were euthanised by exsanguination and cervical dislocation and the reproductive organs (uterus, ovary, vagina) were harvested for morphometric studies and histology.

Vaginal Cytology

A vaginal swab was taken from all eighteen rats and was done using a 1.0 mL Pasteur pipette to introduce about 50 uL of distilled water into the vagina of the rats. The vagina was flushed twice and then its fluid was aspirated and deposited on a glass slide. The fluid was allowed to dry on the slide, fixed with absolute ethanol, stained with Giemsa stain and air dried. The slides were viewed under the microscope to determine the predominant cell type in the smear recorded. The methods of Cora *et al*. (2015) and Singletary *et al*. (2005) were used to classify the oestrous stage into proestrus, oestrus, metoestrus, and dioestrus, and to score as 1, 2, 3, and 4, respectively. A score of '1' was given to proestrus when there was more than 50 % presence of large rounded nucleated (parabasal) cells. oestrus had a score of '2' when polygonial cornified epithelial (superficial) cells were predominant. Score '3' was given to metoestrus when there was an equal presentation of all the epithelial cell types; parabasal, superficial, and leucocytes. Score '4' was given to dioestrus when the smear contained predominantly (more than 50%) leucocytes. All the slides were assessed by an individual who did not know the treatment group of the rats.

Morphometric Studies

The harvested organs: reproductive tract (oviduct, uterus, cervix and vagina) and ovaries were first gently debrided of surrounding connective tissues using a scalpel blade. The reproductive tract and paired ovaries were weighed separately. The weights were expressed as a ratio to a hundred grams of the final body weight (mg/100 g BW): organ weight (mg) / final body weight (g) X 100. After recording the weights, the organs were set in 10% formalin and processed for histology.

Hormone Analysis

The blood samples collected in plain vacutainer tubes were centrifuged at 300 x g for 10 min. The serum was aspirated and analysed for oestradiol, progesterone, and luteinising hormone levels. The hormone analysis was by a chemiluminescence immunoassay analyser (Maglumi 800 Snibe, Shenzhen New Industries Biomedical Engineering Co., Ltd, China) using Maglumi® CLIA kits for oestradiol, progesterone, and luteinising hormones. The laboratory validated the use of the kits for rat serum. The precision, specificity, and sensitivity as per the manufacturer are presented in Table 1.

Table 1: The performance characteristics of the Maglumi® CLIA hormonal test kits

Test kit	Coefficient of Variation		Sensitivity	Specificity
	Intra assay	Inter-assay		
Oestradiol	< 10%	<10%	< 8 pg/ml	99.9%
Progesterone	< 10%	<10%	< 0.13 ng/ml	97.5%- 99.7%
LH	< 6%	<6%	< 0.2 mlU/ml	98.7% - 99.0%

Haematology

The blood samples collected in EDTA tubes were analysed for complete blood count using a 3-part automated haemoanalyzer (Automated haemoanalyzer MC Jefferson LE046D).

Data Analysis

Data analysis was conducted using SPSS version 26 software. A one-way ANOVA was utilized to assess significant differences between groups for the various measured parameters, with a significance level set at $p < 0.05$. When significant differences were found, Turkey's Post Hoc test was employed to identify which means differed significantly at the same $p < 0.05$ level.

In cases where the assumptions of normality and homogeneity of variance were violated, the Kruskal-Wallis H test was implemented to determine significance. Results are presented as means ± standard deviation (SD).

RESULTS

Effect of Oleic Acid on the Oestrous Cycle.

The vaginal cytology of the Wistar rats before and after the experiment is presented in Figure. 1. Oestrous stage scores of 1, 2, 3, and 4 represent proestrus, oestrus, metoestrus, and dioestrus, respectively. 0.5, 1.5, 2.5, 3.5, and 4.5 represent a transition between two stages and represent early proestrus, late proestrus, late oestrus, late metoestrus, and late dioestrus, respectively. There was no difference in oestrous cycle before and after treatment ($p > 0.05$). The variation in the oestrous cycle stage between groups before and after treatment was not significant ($p > 0.05$). All the rats were in the luteal phase before and at the end of the treatment.

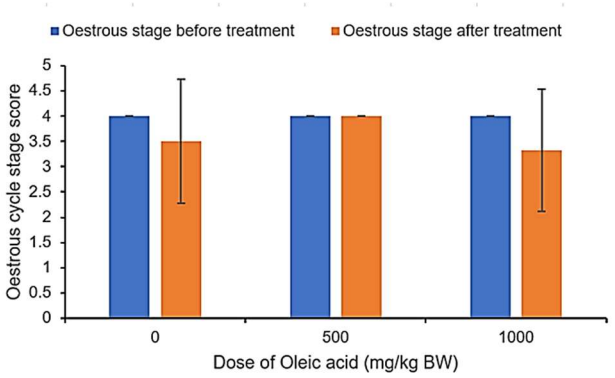

Figure 1: Effect of oleic acid on the oestrous cycle of diet-unrestricted female Wistar rats. $p > 0.05$.

Effect of Different Doses of Oleic Acid on Body Weight

The study showed a significant ($p < 0.05$) decrease in the change in body weights between the control and OA-treated groups (Figure 2). However, no significant change was observed in the initial body weight and the final body weight of the exposed rats.

Effect of Oleic acid on Reproductive Organ Weight

The relative reproductive tract weight of the female rats was significantly different ($p < 0.05$) The reproductive tract weight of rats treated with 1000 mg/kg BW of oleic acid was higher than that of the control group (Figure 3). The relative paired ovarian weight and the final body weight were not significantly altered ($p > 0.05$) by the treatment protocol when compared with the control group.

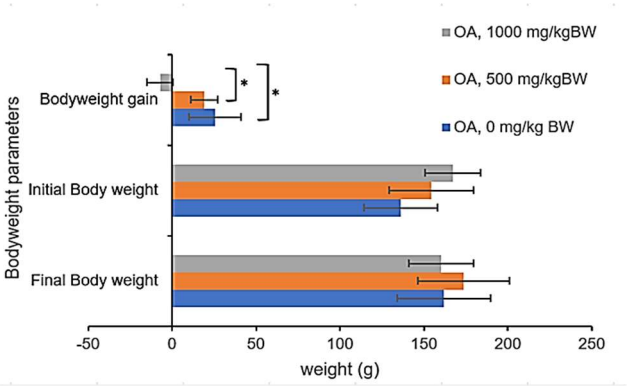

Figure 2: Impact of various oleic acid doses on bodyweight parameters of diet-unrestricted female Wistar rats. *Statistically significant difference ($p < 0.05$). OA= Oleic acid.

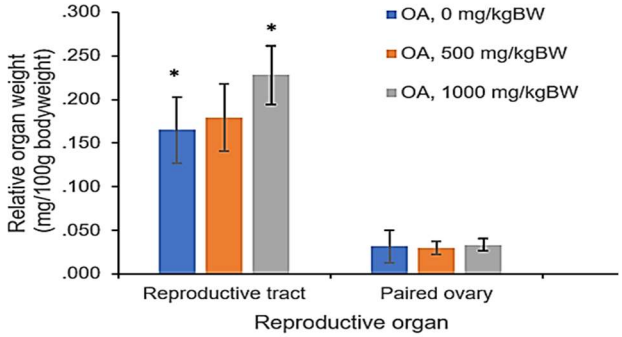

Figure 3: The impact of different doses of oleic acid on the reproductive organ weight of female Wistar rats. *Statistically significant difference ($p < 0.05$). OA= Oleic acid

Effect of Oleic acid on Serum Hormone Levels

The hormonal profiles of the three groups are shown in Figure 4. No significant variation ($p > 0.05$) was observed in oestrogen, progesterone, and luteinising hormone levels among the treatment groups.

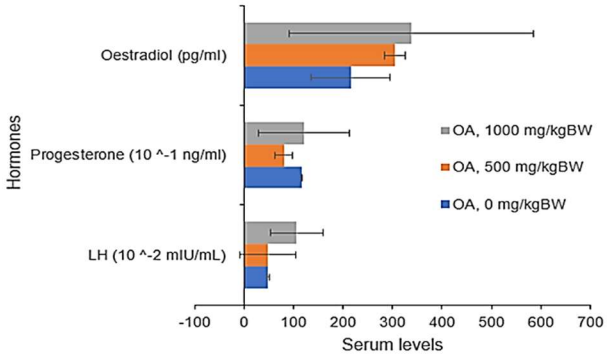

Figure 4: Serum hormone levels of female Wistar rats subjected to varying doses of oleic acid. OA= Oleic acid, n=5

Haematological Effects of Oleic Acid

Erythrogram and thrombogram analysis

The levels of the red blood cell (RBC) parameters (erythrogram) and blood platelets (thrombogram) of the three groups of rats are presented in Table 2. There was no significant difference ($p > 0.05$) in the RBC and Platelet parameters of the three groups of rats after the treatment.

WBC Analysis

The results of the WBC analysis of the three groups are presented in Figure. 5. Oleic acid at 500 and 1000 mg/kg body weight did not alter the total WBC and lymphocyte counts. However, it had a significant effect ($p < 0.05$) on the Mid-cell population (Basophils, eosinophils, monocytes and mast cells) and neutrophils (Figure 5A). There was a dose-dependent increase in Mid-cell counts as the dose of oleic acid increased. The neutrophil count was higher in the 1000 mg /kg body weight group than in the control group. The neutrophil count in the 500 mg/kg body weight group was like the counts in the control and 1000 mg /kg body weight group. When examining the relative proportions of the differential WBCs, it was observed that oleic acid significantly reduced the lymphocyte population but increased the population of mid-cells and neutrophils (Figure 5B). The effect of oleic acid on the lymphocytes, mid-cells and neutrophils population was dose dependent. Notably, the neutrophil population was higher in the 1000 mg /kg body weight group than in the other groups.

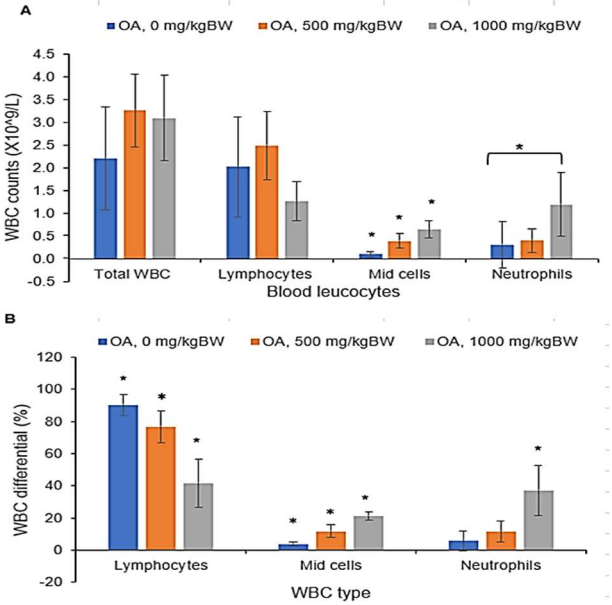

Figure 5: WBC counts and differentials of female Wistar rats treated with various doses of oleic acid. A) Total WBC counts. B) Differential WBC count. *Statistically significant difference ($p < 0.05$). OA= Oleic acid

Table 2: RBC and platelet parameters of female Wistar rats treated with various doses of oleic acid.

Treatment groups	RBC (10^{12}/L)	HGB (g/dL)	HCT (%)	MCV (Fl)	MCH (pg)	MCHC (g/dL)	PLT (10^9/L)
Control	7.812 ± 0.760	15.860 ± 1.484	44.560 ± 4.178	57.120 ± 2.606	20.340 ± 0.902	35.620 ± 0.415	449.00 ± 233.93
500mg/kg	6.336 ± 0.750	12.600 ± 1.249	35.800 ± 2.851	56.667 ± 2.201	19.966 ± 1.550	35.266 ± 2.369	390.00 ± 210.43
1000mg/kg	7.140 ± 0.904	13.900 ± 2.438	40.080 ± 6.160	52.000 ± 7.783	19.360 ± 1.078	34.560 ± 0.934	450.60 ± 199.02

Values are presented as Mean + SD. There was no significant effect of oleic acid on the RBC and platelet parameters of normal cycling female Wistar rats. (n=5, $p > 0.05$)

DISCUSSION

Effect of Oleic Acid on Body Weight Gain and Reproductive Organ Weight

The results indicated that administrating oleic acid at 500 and 1000 mg/kg body weight for 15 days caused a significant decrease in body weight gain. Research has suggested that diets enriched in monounsaturated oleic acid (OA) may have favourable effects on body composition and could aid the management and prevention of obesity. Specifically, emerging evidence highlights the positive role of OA in regulating body weight (Tutunchi *et al*., 2020).

Additionally, treated virgin Wistar rats showed a significant increase in the relative weights of their reproductive tracts compared to the control group after exposure to oleic acid. This finding is particularly important in the context of female reproductive endocrinology and health. An increase in uterine weight is often linked to the influence of oestrogen or oestrogen-like endocrine disruptors (Owens *et al*., 2003; Passoni *et al*., 2021). In the current study, while there was an observed increase in oestrogen levels, it was not statistically significant.

Effect of Oleic Acid on Hormone Levels

The serum oestradiol, progesterone and luteinizing hormone levels of the oleic acid-treated rats did not differ from the control groups in the current study. The findings of this study contrast with those of Yenuganti *et al.* (2016) and Zhou *et al*. (2022). Both Yenuganti *et al*. and Zhou *et al*. reported that oleic acid, when not accompanied by other fatty acids, inhibits steroid hormone production in vitro in cultured granulosa cells, specifically reducing serum oestrogen and progesterone levels. However, in support of our current findings, Baddela *et al*. (2022) observed that unsaturated fatty acids, like oleic acid, do not exhibit an anti-steroidogenesis effect when both saturated and unsaturated fatty acids are present in follicular fluids.

In an in vivo study, Zhou *et al*. (2022) fed mice an oleic acid-rich diet for eight weeks and reported reduced oestrogen and progesterone levels, but they did not disclose the other components of this diet, which may have excluded saturated fatty acids. In the current study, the treated and control rats were given an unrestricted diet, and oleic acid was supplemented in the test group for only 15 days. This unrestricted diet included 6% fats made up of both saturated and unsaturated fatty acids. It is possible that the coexistence of these fatty acids hindered the antisteroidogenic effect of oleic acid in our study.

Additionally, the duration of oleic acid treatment may have contributed to the observed lack of effect on oestrogen and progesterone steroidogenesis and the oestrous cycle. In the study by Zhou *et al*. (2022), the treatment lasted eight weeks, while our study used only 15 days. Another factor contributing to the differences between the two studies could be the sample size because

they used 30 mice per group, whereas we used only 6 rats per group.

The authors recommend further research to examine the antisteroidogenic effects of oleic acid when administered with or without saturated fatty acids, for extended periods, and with larger sample sizes.

Effect of Oleic Acid on Haematologic Profile

The current study evaluated the red blood cell (RBC) and white blood cell (WBC) parameters, and blood platelet levels of both oleic acid (OA)-treated and control rats. All measurements fell within the normal ranges reported by Patel *et al*. (2024), suggesting that OA treatment at the doses and duration used does not impair the health of Wistar rats. However, compared to the control group, oleic acid significantly decreased the proportion of lymphocytes, which participate in specific or humoral immunity. In contrast, it increased the proportion of mid cells (such as basophils, eosinophils, mast cells, and monocytes) and neutrophils, which are essential for the initial, non-specific immune response. Since normal physiological processes can involve either pro-inflammatory or anti-inflammatory responses, oleic acid should be used with caution in these contexts. For instance, in early pregnancy, differentiated monocytes (macrophages) are among the cells at the placental sites and engage in regulating trophoblast invasion, angiogenesis and spiral artery remodelling. Increased infiltration of macrophages to placental sites is associated with abnormal placentation conditions like preeclampsia (Renaud *et al*., 2005; Faas *et al*., 2018). More so, in the current study, oleic acid caused a decrease in the lymphocyte population of leucocytes. The relative lymphocyte count in the OA high-dose group was lower than the normal range for female Wistar rats aged 10-14 weeks, as reported by Patel *et al.* (2024). Lymphocytes, including B cells, T cells, and subpopulations of helper T cells play a crucial role in regulating trophoblast invasion, angiogenesis, and vascular remodelling. Their activity is essential for inducing tolerance to foetal alloantigen and maintaining pregnancy (Pompura *et al*., 2021; Huang *et al*., 2020). Therefore, using oleic acid during this critical period might impede pregnancy.

The results from the normal haematology tests and the absence of recorded deaths among the treated rats suggest that oleic acid, at the current dosage and treatment duration, was safe for them. However, caution is required regarding its effects on the immune and reproductive health of females, as it has the potential to alter reproductive tract weight and affect immuno-reproductive cell populations.

Conclusion

Oleic acid at 500 and 1000 mg/kg body weight has a depressing effect on the lymphocyte population and an enhancing effect on the mid-cell population of immunoreproductive cells of the female Wistar rat model. Oleic acid at the current doses, decreased body weight gain, and increased relative reproductive tract weight, but had no significant effect on oestrogen, progesterone, and LH levels.

Conflict of Interest

The authors have no conflicts of interest to declare.

Authors Contribution

CIC: Conceptualization, Formal analysis, Methodology, Project administration, Resources, Supervision, Validation, Visualisation, Writing- review & editing. SRO: Investigation, Data curation, Resources, Validation, Writing original draft. ECD: Investigation, Data curation, Resources, Validation, Writing original draft. OPC: Investigation, Data curation, Resources, Validation, Writing original draft.

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