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RESEARCH ARTICLE **AJHSE040108**

OXIDATIVE STRESS IN OVARIES OF FEMALE WISTAR RATS EXPOSED TO GEOPHAGIC CLAY (EKO)

[*1]EDENE, O. A. & [1]OGBEIDE, G. E.

[1]Department of Environmental Management and Toxicology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria
***Corresponding Author's E**-mail: osemudiamen.anao@uniben.edu Phone: +2347061857637*

ABSTRACT

The impacts of geophagy on reproductive health, especially the ovaries remain unexplored. The reproductive health of individuals (mostly pregnant women) may be impacted, by exposure to geophagic clay, as there is evidence to substantiate the presence of some contaminants in the studied geophagic clay. The ovary which is the site of production of female egg cells, in the female reproductive system can give useful information on the reproductive effects of this practice in exposed organisms. In this study, thirty-six (36) healthy adult female rats of the Wistar strain, were placed in six (6) groups, of six (6) animals each (A-F) and administered geophagic clay orally with gavage, for forty-two (42) days. Group (A) which served as the control group received feed and distilled water only. Groups (B-F) were administered (250, 500, 1000, 1500, and 2000) mg/kg bw geophagic clay dissolved in distilled water. Markers of oxidative stress were assessed. Histopathological examinations were also carried out on the ovaries. Comparison of the results obtained, by matching control group with the treatment groups, revealed that oxidative stress markers [superoxide dismutase (SOD) and malondialdehyde (MDA)] were elevated significantly ($p < 0.05$) mostly in the groups administered high doses (1000-2000 mg/kg bw) of geophagic clay. There was a marked depletion ($p < 0.05$) in reduced glutathione (GSH) level (2000 mg/kg bw). Histopathological examination of the ovaries revealed slight alterations in the anatomic features predominantly in the group administered the highest dose (2000 mg/kg bw). This finding infers possible toxicity in the ovaries especially when geophagic clay is consumed in high concentrations. This implies that geophagic clay may cause a redox imbalance in the ovary, altering female reproductive function, especially when consumed in high doses.

Keywords: *Contaminants, 'Eko', Pregnant women, Reproductive health, Reproductive system, Toxicity.*

INTRODUCTION

Prostate Geophagy, the practice of consuming earthy substances, transcends cultural boundaries, as it is a practice rooted in cultural and traditional beliefs (Kambunga *et al*., 2019; Kimassoum *et al*., 2023). Geophagic clay is traditionally called "eko" in Edo (Eigbike *et al*., 2022; Edene and Aghedo, 2023). Women, men, and children have been reported to partake in its consumption (Caillet *et al*., 2019; Mogongoa, 2020). Communal motives for geophagia practice include the belief that it helps in remedying inconveniences that arise from diarrhea, it is a means of assuaging nausea and vomiting in pregnant women, as well as a strong belief that it enhances fertility (Macheka, *et al*., 2016, Phakoago *et al*., 2019).

Due to the wide range of participants in this habit, it is important to understand its impending impact on human health. Most works of literature have reported exclusively on the constituents of geophagic clay. It is said to contain minerals such as iron, calcium, and magnesium which play a role in nutrient supplementation. Studies have also shown that it may also be contaminated with heavy metals, microorganisms, helminths, and pesticides (Orisakwe *et al*., 2020; Getachew *et al*., 2021; Adewale *et al*., 2022; Davies, 2023; Edene and Aghedo, 2023). The Ubiaja geophagic clay specifically, contains heavy metals such as cadmium, lead and chromium at concentrations above the WHO permissible limits (Edene and Aghedo, 2023), which may be ingested in geophagy, thereby resulting in maternal and /or fetal death (Mashao *et al*., 2021; Nabuuma, 2021). Hence a potential risk factor for exposed individuals. Some pregnant women especially in Africa, indulge in geophagia (Israel *et al*., 2019; Kambunga *et al*., 2019). This raises unease due to the probable risks to both mother and the developing fetus (Kambunga *et al*., 2019; Kortei *et al*., 2020). Furthermore, geophagy may interfere with nutrient absorption, potentially exacerbating maternal nutrient deficiency.

The ovaries are a vital component of the female reproductive system and are responsible for the release of eggs (ova) for fertilization and secretion of female sex hormones (estrogen and progesterone) (Haeberle, 2020). However, the ingestion of geophagic clay, which may expose individuals to toxic substances, can potentially lead to adverse effects on the ovaries and, consequently, impact the entire female reproductive system. Scientific research has established a connection between geophagy and heavy metal toxicity (Lar *et al*., 2015, Odongo, *et al*., 2016). It has been established that even at very low amounts of exposure to heavy metals, they could have very debilitating effects. This is due to their extensive distribution in the environment and the accumulative potential of these metals in specific tissues of the human system (Lar *et al*., 2015, Odongo, *et al*., 2016, Kambunga *et al*., 2019). Specific heavy metals including, but not restricted to, lead, mercury, and arsenic have a high toxic potential at very minute concentrations of exposure, hence are invariably of no use in human physiological processes (Fu and Xi, 2020). In ovarian physiological metabolism, antioxidants and reactive oxygen species (ROS) are seen to play important and key roles in modulating the physiological processes taking place in the ovaries specifically, and ultimately in the female reproductive system (Goutami *et al*., 2022, Didziokaite *et al*., 2023).

Previous studies have shown that in different luteal phases of ovarian physiology, activities such as folliculogenesis, ovulation, fertilization, placental growth, embryogenesis, and implantation are affected greatly by the activities of antioxidants and reactive oxygen species (ROS) (Bhardwaj *et al*., 2021). To support normal ovarian function, maintaining the redox balance is very vital and antioxidants have been seen as critical in achieving this. Against this background, assessing oxidative stress levels in the ovaries on exposure to geophagic clay is vital in understanding safety issues regarding its consumption. Hence, this study seeks to investigate changes in redox state

of the ovary by examining related markers and histopathology in Wistar rats exposed to varying concentrations of geophagic clay (eko).

MATERIALS AND METHOD

Sample collection

Geophagic clay administered to the Wistar rats was purchased from three (3) different locations in Ubiaja, Edo State, Nigeria. They included: Ebhohimi market, Ewato market, and market stores around Ubiaja correctional facility (6 º 6501803N, 6 º 3902683E; 6 º 5068798N, 6º 3327434E and 6 º 65972N, 6 º 38222E respectively). Ziplock bags were used to pack the samples to reduce exposure levels to contaminants. Subsequently, the samples were crushed into fine powder under aseptic conditions and used for the experiment.

Reagents

Chloroform, picric acid, glycerol, adrenaline, hydrochloric acid, carbonate buffer, sodium hydroxide, pyrogallol, phosphate buffer, hydrogen peroxide, potassium permanganate, sulphuric acid, thiobarbituric acid (TBA), trichloroacetic acid (TCA), formaldehyde and Ellman's reagent. The reagents used were purchased from the British Drug House (Poole, Dorset, UK) and they were certified to be of good analytical standard.

Acclimatization

Adult female rats weighing 150–200 g were bought from the Animal House of the Department of Anatomy, University of Benin, Edo State, Benin City. They were accustomed to the new environment for 14 days and housed in well-ventilated cages, fed rat pellets and water spontaneously, and naturally exposed to light and dark for 12 hours each (El-Ashmawy *et al*., 2022).

Study design

Rats were placed into six groups of six (6) rats each and administered geophagic clay orally by gavage for forty-two (42) days. Group 1 (control rats) received only distilled water orally, while groups 2, 3, 4, 5, and 6 received geophagic clay (250, 500, 1000, 1500, and 2000 mg/kg bw) orally.

Prior to the main study, we carried out an acute toxicity test, using the Lorke method (Lorke, 1983) in which female rats were exposed to geophagic clay concentrations of 10, 100, 1000, 1600, 2900, and 5000 mg/kg bw. The LD50 was found to be > 5000 mg/kg bw. Therefore, in the main experiment, we chose a dose below 5000 mg/kg, which corresponds to the average daily intake concentrations as obtained from Luoba *et al*. 2004.

Determination of sample intake

The doses to be administered were determined using the formulae below in relation to the weight of the rats

$$\text{Volume (mL)} = \frac{\text{Dose (mg/mL) x animal weight (g)}}{\text{Concentration of Stock (mg/mL)}}$$

The volume administered to each rat was calculated using the formula:

Volume = dose × weight/ Conc. of stock

Stock concentration (10%): 10 g of the geophagic clay dissolved in 90 ml of distilled water**.**

Sacrifice of experimental animals

The technique employed in sacrificing the rats was by cervical dislocation. The organ of interest which was the ovaries were collected and made clean by washing in 1.15% KCl buffer stored at a temperature of 4 ^{o}C (Wasek *et al*., 2018).

Preparation of the post-mitochondrial fraction

The organs were prepared for analysis by introducing the organs into a Potter-Elvehgen homogenizer and homogenized in 0.1 M phosphate buffer with a pH of 7.4, following the method described by Abiola *et al*., (2019). After homogenization, the resulting product was subjected to centrifugation at 10,000g for 10 minutes, using a cold centrifuge set at 4 ^{o}C. This process allowed the separation of supernatants (post-mitochondrial fractions), which were used for evaluating markers of oxidative stress.

Estimation of Total protein concentration

The biuret reaction method as described by Gornal *et al*. 1949 was used in the estimation of Total protein concentration. The test sample (0.05 mL) was placed in a test tube, with the addition of 0.05 mL of the standard and 1.5 mL of the biuret reagent. To the blank, 1.5mL of Biuret and 0.05 mL of purified water were added. The tubes were shaken intermittently to ensure uniformity and subjected to heating in a water bath at 37 ^{o}C temperature for about 10 minutes. Using a spectrophotometer they were read at a wavelength of 546 nm against the blank.

Estimation of Superoxide dismutase (SOD)

The method of Misra and Fridovich (1972) was utilized in the determination of Superoxide dismutase (SOD). The preparation of the standard was done by putting 2.7 mL of 0.05 M carbonate buffer in a test tube. A tube containing the test sample was prepared by placing 0.2 mL of the sample into the tube, then adding 2.5 mL of carbonate buffer. A standard was prepared by placing 2.7 mL of 0.05 M carbonate buffer in a test tube. Then, 0.3 mL of 0.3 mM epinephrine was added to each test sample and standard tube to start the reaction. Readings were taken every 1 min at 480 nm.

Estimation of catalase activity

The method according to Cohen *et al*. (1970) was used. This estimation is hinged on the ability of hydrogen peroxide to decompose when the material containing the enzyme is added. Excess potassium permanganate (KMNO4) is reacted with tissue homogenate and the resulting hydrogen peroxide from this reaction is determined with the aid of a spectrophotometer at 480 nm via quantification of the residual $KMNO_4$. To a known volume of plasma (0.5 mL), 5.0 mL of H_2O_2 was added. The resulting product was shaken together to allow for a uniform mixture, and then stood for 30 mins. In order to bring the reaction to a halt, 1.5 mL of 6 M H2SO4 and 7 mL of 0.01 M $KMn0_4$ was added. Distilled water was used as blank. The mixture in the tube was read in a spectrophotometer at 480 nm within 30 – 60 seconds against the blank to get the absorbance. The enzyme activity was expressed as µmoles of H2O2 composed/min/mg/protein.

Estimation of Glutathione peroxidase (GPx) activity

Glutathione peroxidase enzyme activity was determined by the method described by Rotruck *et al*. (1973) with minor modifications. To a test tube containing phosphate buffer (6 mL), 1.5 mL of sodium azide (NaN_3) was added, followed by the addition of 2.5 mL of glutathione, subsequently, 0.05 mL of hydrogen peroxide was added and then 0.25 mL of the sample. The mixture was incubated in a temperature regulated bath at 37 ^oC for 3 mins; 0.25 mL of TCA was added to make up the final mixture. It was then centrifuged for 5 mins at 3000 rpm. I mL of the supernatant was taken out and mixed with $K2HPO_4$ (2 mL) and DTNB (1 mL). The absorbance was taken at 412 nm against reagent blank.

Estimation of Reduced glutathione

Reduced glutathione was determined by the Ellman method (Ellman, 1959). Ellman's reagent was used in determining ovarian GSH levels. The test sample (200 µl) was double diluted with the addition of 5% TCA. The TCA added was solely to cause a precipitation of the protein content of the mixture. The mixture was then centrifuged at 10,000 g for 5 minutes to obtain a supernatant. To the supernatant obtained, DTNB solution (Ellman's reagent) was added and the absorbance reading taken at 412 nm.

Estimation of Lipid peroxidation

Lipid peroxidation levels in tissues are measured as concentrations of Malondialdehyde (MDA) formed. This was determined using the thiobarbituric acid assay (Buege and Aust, 1978). The sample (2 mL), was placed in a test tube, and glacial acetic acid (2 mL) was added, followed by the addition of 1% thiobarbituric acid (2 mL). A stopper was placed on the test tube loosely and it was dipped in steaming water for fifteen minutes during which it was shaken intermittently. The mixture obtained was allowed to cool and centrifugation was done at 3000 rpm for 10 minutes. It was then read at 532 nm against the reagent blank.

Statistical analysis

Graph pad Prism 5 software was employed in the statistical analysis of the results. A grouped table option was used to enter replicate values of the data, in side-by-side columns. One-way analysis of variance (ANOVA) was used in comparing the results from the groups treated with geophagic clay against the control groups and the post-HOC test employed was the Dunnett's multiple comparison test.

RESULTS

Effects of "eko" consumption on Total protein (TP) in ovaries of adult female Wistar rats.

The outcomes following the administration of geophagic clay to female Wistar rats are illustrated in Figures 1-6. Figure 1 demonstrates noteworthy alterations ($p < 0.05$) in the total protein concentrations among the groups treated with geophagic clay doses ranging from 1000-2000 mg/kg bw. Despite a decrease in total protein concentration in treatment groups 1 and 2, compared to the control group, these changes were not statistically significant ($p > 0.05$).

Effects of "eko" consumption on Superoxide dismutase (SOD) in ovaries of adult female Wistar rats.

The findings depicted in Figure 2, demonstrate significant increases ($p < 0.05$), in SOD enzyme activity within the

groups treated with geophagic clay at concentrations ranging from 500-2000 mg/kg, when compared to the control group.

Effects of "eko" consumption on Superoxide dismutase (SOD) in ovaries of adult female Wistar rats.

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Effects of "eko" consumption on Catalase (CAT) activity in ovaries of adult female Wistar rats.

Catalase activity was seen to increase in the result presented in Figure 3 dose-dependently but this increase was insignificant ($p > 0.05$) when compared with control in all the treatment groups.

Effects of "eko" consumption on the activity of Glutathione peroxidase (GPx) enzyme in ovaries of adult female Wistar rats.

The results presented in Figure 4, revealed that GPx activity was increased in groups administered 250-1000 mg/kg of geophagic clay. This trend was different as the concentration increased with a lessening of the enzyme activity in the 1500-2000 mg/kg bw treated groups. Notably these changes were not significant ($p > 0.05$).

Effects of "eko" consumption on Reduced Glutathione (GSH) levels in ovaries of adult female Wistar rats.

Figure 5 demonstrates a decline in reduced glutathione levels with increasing concentrations of the administered geophagic clay. The observed decrease was statistically significant ($p < 0.05$) only in the group that received the highest concentration of geophagic clay. This suggests that the highest dosage of geophagic clay had a notable impact on reducing the levels of reduced glutathione, while lower concentrations did not show significant effects on glutathione levels in comparison to the control group.

Effects of "eko" consumption on Malondialdehyde levels in ovaries of adult female Wistar rats.

The result shown in Figure 6 revealed that Malondialdehyde levels were elevated significantly ($p < 0.05$) in the groups administered 1000-2000 mg/kg of geophagic clay.

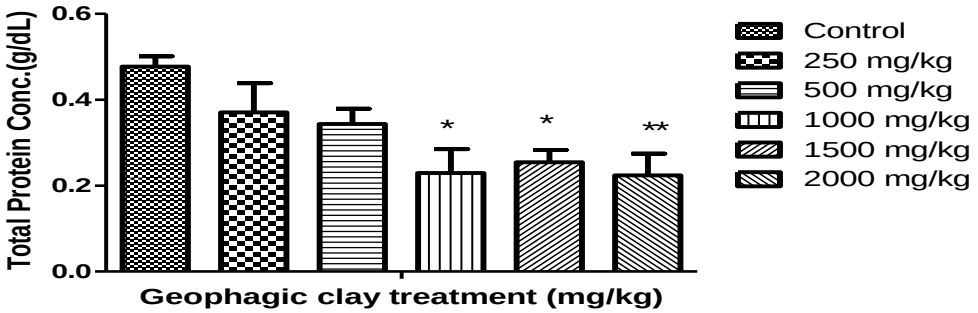

Figure 1: Effects of "eko" consumption on total protein (TP) activity in ovaries of adult female Wistar rats. Data are presented as Mean ± SEM (n=6); *Significant as compared with control; ($p < 0.05$), **More significant ($p < 0.05$).

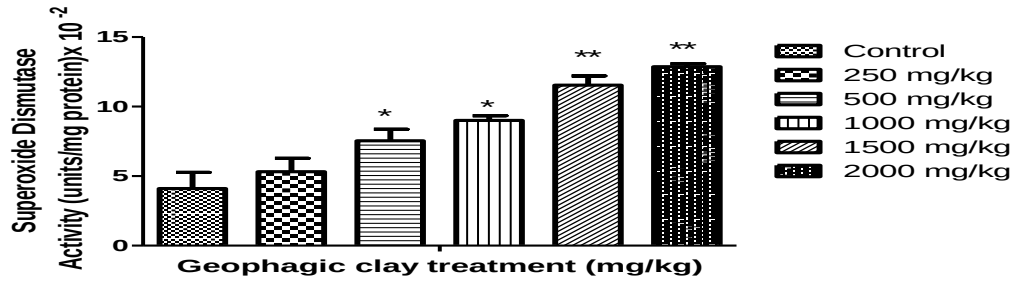

Figure 2: Effects of "eko" consumption on SOD activity in ovaries of adult female Wistar rats. Data are presented as Mean ± SEM (n=6); *Significant as compared with control; ($p < 0.05$), **More significant; ($p < 0.05$).

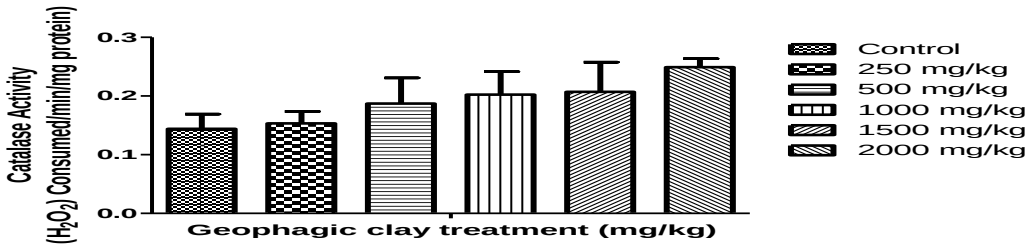

Figure 3: Effects of "eko" consumption on CAT activity in ovaries of adult female Wistar rats. Data are presented as Mean ± SEM (n=6); *Significant as compared with control; ($p < 0.05$), **More significant; ($p < 0.05$).

Figure 4: Effects of "eko" consumption on GPx activity in ovaries of adult female Wistar rats. Data are presented as Mean ± SEM (n=6); *Significant as compared with control; ($p < 0.05$), **More significant; ($p < 0.05$)

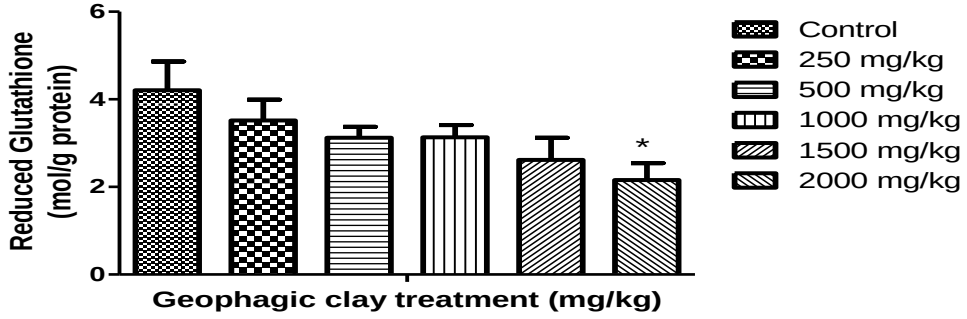

Figure 5: Effects of "eko" consumption on GSH activity in ovaries of adult female Wistar rats. Data are presented as Mean ± SEM (n=6); *Significant as compared with control; ($p < 0.05$), **More significant; ($p < 0.05$).

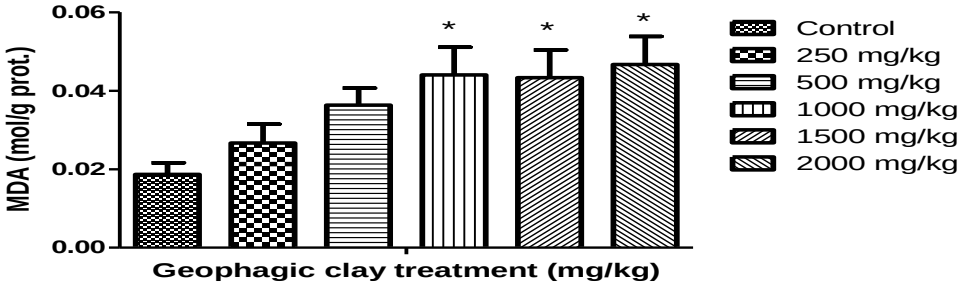

Figure 6: Effects of "eko" consumption on MDA levels in ovaries of adult female Wistar rats. Data are presented as Mean ± SEM (n=6); *Significant as compared with control; ($p < 0.05$), **More significant; ($p < 0.05$).

Histology of the ovary after treatment with geophagic clay

Histological evaluation, revealed mild changes in the ovarian architecture of the rats treated with geophagic clay. There were moderate stromal congestions in the blood vessels indicating an increased blood flow. This result reveals that though there were mild changes in the ovarian architecture, there were no significant damages to the structures of the ovary especially in the groups exposed to low doses (250-500 mg/kg) of geophagic clay.

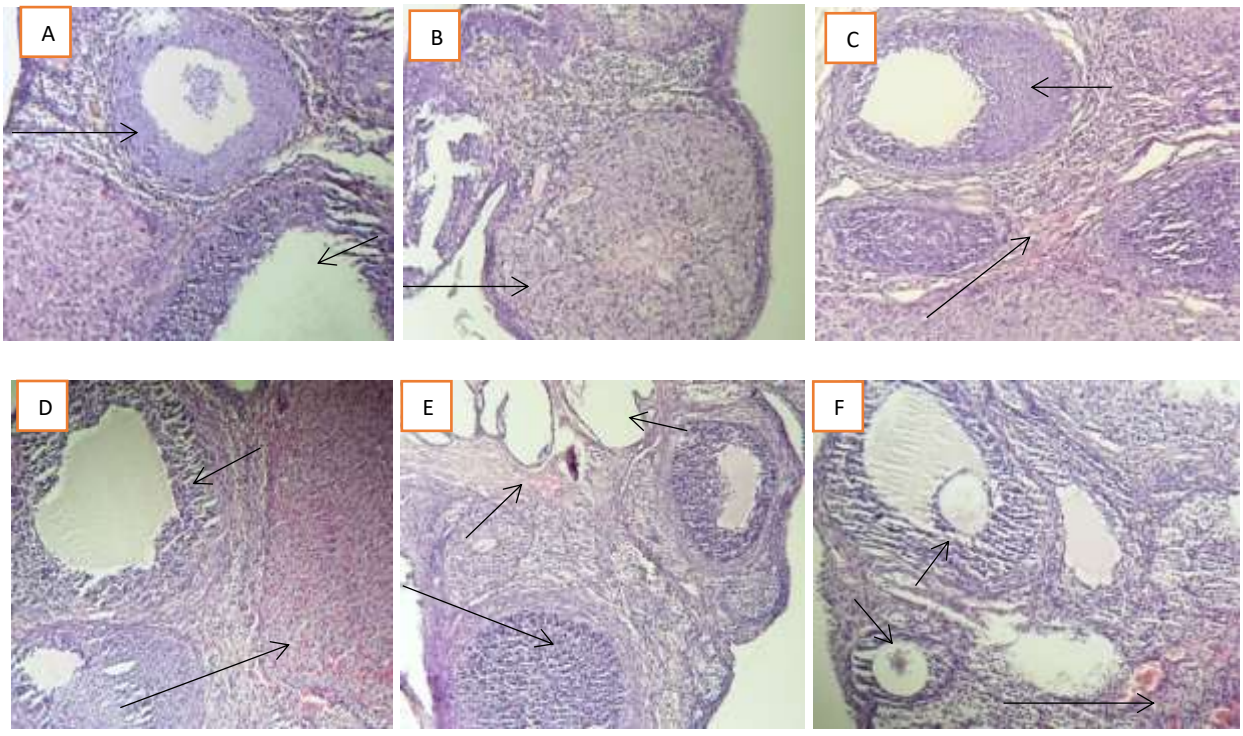

Plate 1: Haematoxylin and eosin stained ovary sections

A) CONTROL: Prominent corpus luteum (long black arrow), normal zona granulosa (short black arrow). B) 250 mg/kg: Leuteinized stroma (long black arrow) C) 500 mg/kg: the blood vessels in the stroma are congested indicating an increased blood flow (long black arrow), Prominent corpus luteum (short black arrow) D) 1000 mg/kg: Stromal infiltrates of eosinophils (long black arrow), mild erosion of the corpus luteum (short black arrow) E) 1500 mg/kg: thickened corpus luteum (long black arrow), slight stromal congestion (short black arrow) and edema F) 2000 mg/kg: increased stromal congestion, erosion, blood accumulation (long black arrow), increased number of follicles (short black arrow) (H&E x 40).

CONCLUSION

Geophagic clays as documented in numerous scientific studies, have been found to contain harmful heavy metals, including arsenic, lead, mercury, and cadmium (Orisakwe *et al*., 2020; Olajide-Kayode *et al*., 2023; Olisa *et al*., 2023). In our preliminary investigation, we confirmed the presence of cadmium, lead, and chromium in the Ubiaja geophagic clay (Edene and Aghedo, 2023). Heavy metals have been implicated in the initiation and progression of certain diseases (Briffa *et al*., 2020 and Jiang *et al*., 2020). Oxidative stress has also been linked to the pathophysiology of these diseases (Zheng,*et al*., 2020, Paithankar *et al*., 2021, Pisoschi *et al*., 2021). In this study, we assessed critical markers of oxidative stress in female Wistar rats exposed to various concentrations of geophagic clay. As depicted in Figure 2, we observed a substantial ($p < 0.05$) and dose-dependent increase in the activity of superoxide dismutase (SOD) in geophagic clay-treated female Wistar rats. This suggests that the elevation in SOD activity may be a response to the generation of free radicals resulting from exposure to different concentrations of geophagic clay. Notably, these findings align with results from other studies wherein SOD concentrations were found to increase upon exposure to different toxicants (Xue *et al*., 2022, Bhat *et al*., 2023, Yapca *et al*., 2023).

The activity of catalase (CAT) enzyme as shown in Figure 3, was seen to increase dose-dependently with increasing doses of geophagic clay treatment. The observed increase was statistically insignificant ($p > 0.05$) when the treatment groups were compared with the control group. This may be an adaptive response to countering the free radical generated by the action of the SOD enzyme. This result is similar to that of Gupta *et al*. (2021), in which an insignificant increase in CAT activity in the testis of male Wistar rats exposed to copper for 24 days was reported. Nwauche *et al*. (2021) also reported a significant increase in CAT activity in Wistar rats fed edible clay after mixture with their feed.

There were observed changes in GPx enzyme activity in all the geophagic clay-treated groups, different from the enzyme activity in the control group. This observed difference was statistically insignificant ($p > 0.05$), as shown in Figure 4. Although the changes seen were insignificant, it gives information on the response to the different concentrations of the geophagic clay. Tazari *et al*. (2018) in their study reported that relative to their control animals, the activity of the glutathione peroxidase enzyme was significantly reduced in the animals treated with arsenite, which resulted from the enzyme being used up in response to the generation of free radicals. Though this was contrary to our results, the observed changes in our study could be explained as an initial adaptive response to the generation of free radicals on exposure to geophagic clay at different concentrations.

Glutathione is a powerful antioxidant that combats free radicals; it is depleted in conditions of oxidative stress (Raj Rai *et al*., 2021). Assessment of glutathione levels in the ovaries of exposed Wistar rats revealed a decline in GSH concentrations in the geophagic clay treated group's dose-dependently, which was significant in the group that was administered the highest concentration of geophagic clay (Figure 5). This is indicative of increased free radical generation causing depletion in glutathione levels. This result is similar to the finding of Anyanwu *et al*. (2020) where there were significant elevations in the GSH activity in the kidney of rats co-administered with a low dose of a combination of different heavy metals (lead, cadmium, and mercury) indicating an alteration in the redox balance of the exposed organism. The behavior of our geophagic clay-treated rats in this manner, therefore, indicates its ability to alter the redox balance in the studied organ (ovary).

Malondialdehyde (MDA) is a principal end-product of lipid peroxidation (Gęgotek, and Skrzydlewska, 2019). Malondialdehyde (MDA) is produced when reactive oxygen species degrade polyunsaturated lipids. Oxygen

species that are reactive can break down polyunsaturated lipids and produce a substance known as MDA; hence it's a potent marker of oxidative stress in tissues. This study revealed increased levels of MDA in exposed rats, significantly so in the groups administered higher concentrations of the geophagic clay (1000-2000 mg/kg bw). Apiamu *et al*. (2023), in their study, had similar results from the exposure of rats to a synergistic interaction between some heavy metals ($p < 0.05$) in both liver and renal tissues. A slight raise in MDA levels was the finding of Agomuo *et al*. (2019), in their study on exposure of gestational rats to single doses (500 mg/kg body weight) of clay beverage, culminating in oxidative stress. Hence the observed elevation in MDA levels in groups administered high doses (1000-2000 mg/kg) of the geophagic clay used in this study is indicative of lipid peroxidation in the ovaries of the treated rats.

Morphological studies in haematoxylin and eosin stained ovary sections (Plate 1), revealed slight alteration on the anatomic features of the ovary. The corpus luteum was maintained in the treatment groups administered low doses of geophagic clay when compared with control (250-500 mg/kg bw). In the groups administered higher doses, there were stromal infiltrates of eosinophils, mild erosion of the corpus luteum (1000 mg/kg bw), thickened corpus luteum, slight stromal congestion, with thickness of ovarian surface structures (germinal epithelium and zona granulosa) and edema when compared with control (1500 mg/kg bw). Also increased stromal congestion and blood accumulation was seen in the group administered 2000 mg/kg bw). Thus indicating possible impairment in functions as a result of alterations in the normal ovarian architecture mostly in the groups administered higher doses. Similar findings has been reported by Nwauche *et al*. (2021) in their study in which Wistar rats were fed edible clay (Nzu) at varying concentrations for 24 days particularly in the group exposed to the highest treatment concentration.

During oxidative stress related diseases, there are usually changes in the levels of antioxidant markers (Chen *et al*., 2020). An elevation or depletion in some of these markers gives an indication of the safety of the xenobiotic to which the organisms have been exposed. Overall from this study, we can say that exposure to higher concentrations of geophagic clay may cause oxidative stress in the ovaries of exposed organisms and by implication may have undue detrimental effects on the reproductive health of exposed species.

CONCLUSION

Results from this study revealed that there may be possible alterations in ovarian function via oxidative stress mechanism especially in the groups administered higher doses of geophagic clay. Taking an overview of the biochemical and morphological parameters assessed in the ovary, it can be said that exposure to high doses of geophagic clay, caused oxidative stress in the ovaries, but exposure to low doses caused changes that could be described as adaptive. Hence, its consumption should be discouraged especially in women of reproductive age.

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CONFLICT OF INTEREST

The authors unanimously declare that there is no skirmish of interest whatsoever.

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