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Cucurbita maxima Seed Oil Pre-treatment Ameliorates Ovarian Oxidative Stress-mediated dysfunction Associated with Diclofenac therapy

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Abstract

BACKGROUND AND AIM: Frequent use of diclofenac provokes oxidative stress that may compromise reproductive health. Anti-oxidants present in *Cucurbita Maxima* Seed Oil (CMSO) may offer therapeutic relief.

METHODOLOGY: Twenty-four randomized rats were used. Group A was control. Group B received only 100mg/kg of Diclofenac sodium intraperitoneally (day 22 to 24). Groups C and D were pre-supplemented with 2ml/kg and 4ml/kg of CMSO respectively (day 1-24) before toxic dose of diclofenac at specified days. The rats were sacrificed; blood samples were used for assay of Malondialdehyde (MDA), Catalase (CAT), Progesterone (PROG) and ovaries processed for H and E histology.

RESULTS: Malondialdehyde (MDA) activity was significantly ($p < 0.05$) increased in Diclofenac alone treated rats (group B) when compared to Control. This elevation in MDA suggests excessive lipid peroxidation. Similarly, Progesterone (PROG) level was significantly ($p < 0.05$) increased in Group B compared to control. Hyper secretion of Progesterone may inadvertently affect conception via negative feedback pathway by inhibiting endometrial receptivity and decidualization. It was observed that rats given high dose CMSO (Group D) showed significantly decreased MDA with a reduction in Catalase (CAT) level after scavenging reactive metabolites of diclofenac when values were compared to group B at $p < 0.05$. This is believed to be due antioxidants and poly saturated fatty acid content in CMSO which preserves ovarian cellular membrane from free radical attack by inhibiting spontaneous cell membrane depolarization and minimizing amount of drug reactive metabolite reaching ovarian tissue by nourishing endothelial membrane barrier. Group B rats showed degeneration in corpus luteum (CL), compared to control and high dose CMSO pre-treated rats.

CONCLUSION: CMSO is of therapeutic benefit against diclofenac-induced ovarian dysfunction.

Keywords:

Cucurbita maxima Seed Oil; Diclofenac; Ovarian Toxicity; Toxicity; Oxidative stress

INTRODUCTION

The ovaries are paired sex glands or gonads that are concerned with female reproductive cycle and steroidogenesis (Neeglund and Panchaksharaya, 2016). The growing consumption of non-steroidal anti-inflammatory drugs (NSAIDs) like ibuprofen, aspirin and diclofenac obtained by 'over the counter' prescription has raised public health concerns (Kristensen *et al.*, 2018). Like other countries, diclofenac is widely distributed in Nigeria as sodium or potassium tablets, injectable or sprays. It is mostly available in small pharmacies and drug patent shops under different trade names (Altman *et al.*, 2015). Diclofenac is commonly used as an effective analgesic against menstrual cramps and endometriosis (Derry *et al.*, 2009) Although NSAIDs like diclofenac provide symptomatic relief from pain and swelling in chronic joint diseases, they can

significantly inhibit ovulation in women through oxidative stress pathway (Hickey *et al.*, 2001; Sherif *et al.*, 2014)

Medicinal plants and plant extracts with anti-oxidant activities have been used to prevent or treat various pathologies related to oxidative stress (Hassan *et al.*, 2017; Anyanwu and Okoye, 2017). This can be attributed to their insignificant side effects, low cost and availability. Thus, minimizing adverse drug reactions/toxicities of NSAIDs could be achieved by exploring natural products. Supplementations of seed oils extracted from different species of pumpkins such as *cucurbita maxima* and *pepo* are natural antioxidants; rich in nutritional and phytochemical compounds such as proteins, minerals, polyunsaturated fatty acids, α - and γ -tocopherol, carotenoids, terpenoids, sterols and quercetin

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(Procida *et al.*, 2013; Amuta *et al.*, 2014; Akin *et al.*, 2018). More recently, a huge interest in a class of triterpenoids; cucurbitacins, has been stated in cucurbita genus while x-raying the correlation between their pharmacological potentials and bioactive composition (Saleh *et al.*, 2019).

Although extracts of *Cucurbita pepo* and *Cucurbita maxima* are the most commonly used *Cucurbita* plants in traditional medicine, the health benefits of *C. maxima* seed extracts appear to be well-documented (Nishimura *et al.*, 2014; Motti and Motti, 2017). However, there is a dearth of information in literature on the medicinal benefit of pumpkin (*cucurbita maxima*) seed oil in combating oxidative stress mediated ovarian toxicity due to acute exposure to Diclofenac, hence this study became germane to investigate *C. maxima* therapeutic role in NSAIDs related ovarian dysfunction.

MATERIALS AND METHODS

Ethical Approval

Ethical approval was obtained from the Animal ethics and research committee of College of Medical Sciences, Alex Ekwueme Federal University Ndufu Alike Ikwo (AE-FUNAI) with no AEFUNAI/ANA/2021/287.

Drug and Chemicals

Cucurbita maxima oil seed (CSMO) was sourced from Hemani herbal company, Pakistan. Diclofenac (DIC) sodium under brand name Diclofen, manufactured by Biochem pharmaceuticals, Mumbai, Maharashtra, India, was used. All other reagents and assay kits purchased were of commercial and analytic grade.

Animals

Twenty four (24) Wistar rats (150g-200g) were purchased and housed in the animal facility of Alex Ekwueme Federal University Ndufu Alike Ikwo, Ebonyi State (AE-FUNAI), Nigeria. The animals were kept in well ventilated cages under normal conditions (12 hr light/12 hr dark) at $25 \pm 3^\circ\text{C}$, fed with rat chow (Vital Feeds Nigeria Ltd., Jos, Nigeria) and distilled water *ad libitum*. The rats were allowed to acclimatize for two week before experimental treatment which lasted for 24 days. Experimental procedures and animal handling were in conformity with international, national and institutional guidelines for the care and use of laboratory animals in biomedical research as promulgated by the Canadian Council of Animal Care (CCAC, 1985).

Experimental design

The rats were randomly divided into four groups (A-D) of six (6) rats each. The Groupings and treatments were as follow: Group A was used as negative control and were orally administered normal saline daily throughout the experimental period (24 days). Group B served as acute toxicity group (positive control). The rats received normal saline as vehicle and intraperitoneal dose of diclofenac (100 mg/kg body weight/day, i.p.) on the 22nd, 23rd and 24th days (last three days) of the experiment (Bolat and Selcuk

2013; Famurewa *et al.*, 2020). Group C were pre-treated with oral doses of 2ml/kg body weight of CMSO for 24 days, followed with toxic dose of Diclofenac on the last three days of the experiment. Group D rats were pre-treated orally with 4ml/kg body weight of CMSO for 24 days, before dose injection of diclofenac. The treatment schedules were adapted from a previously described protocol (Bolat and Selcuk 2013; Famurewa *et al.*, 2020). The dosages of *Cucurbita maxima* seed oil (CMSO) administered in this present study are of considerable safety margin as contained in manufacturer Safety data sheet and therapeutic benefit as established in previous study (Mollika *et al.*, 2020).

Animal Sacrifice

At the end of the experiment, the animals were fasted overnight and sacrificed by cervical dislocation. Blood samples of each rat were immediately collected via cardiac puncture using a 21G needle mounted on a 5ml syringe (Hindustan Syringes and Medical Devices Ltd., Faridabad, India). The syringe was inserted into the heart based on prior palpation of the apex beat. The aspirated blood were introduced into plain sample bottles, centrifuged at 3000 rpm for 15 minutes and the serum extracted for biochemical assays of Progesterone (PROG). The abdomino-pelvic area was carefully dissected; the pair of ovaries was harvested and a part quickly fixed in Bouin's fixative for histological examination. The other ovary was washed in cold saline solution, and blotted on filter paper. The homogenization of the ovary was done in phosphate buffered saline (0.1 M, 1:5 w/v, pH 6.4) and centrifugation was done at 4000 rpm for 20 min. The supernatant was separated for evaluation of Catalase (CAT) and Malondialdehyde (MDA) level.

Biochemical Analyses

The evaluations were done using assay kits from RANDOX, as guided by manufacturer's instruction. Assay of MDA as an index of Lipid peroxidation was estimated colorimetrically by measuring the level of thiobarbituric acid-reactive substances (TBARS) using a previous descriptive method (Wallin *et al.*, 1993). Assay of catalase (CAT) was done using a described method (Sinha, 1972). Serum progesterone concentration was determined by ELISA Kit manufactured by Alpco Diagnostics, Salem, USA following a specified protocol (Abraham, 1981).

Histopathological evaluation

Each fixed ovary was processed using MTP-E Series stainless steel automatic tissue processor manufactured by Medimeas, India. Serial sections (5µm thick) of paraffin embedded tissue were hydrated in decreasing grades of ethanol, oven-dried and stained with haematoxylin and eosin (H&E) dye. The slides obtained were viewed under a microscope to examine histological features and alterations. Relevant areas were then photo-micro graphed and interpreted.

Statistical Analysis

Analysis was done using (SPSS)/ PC computer program (version 23.0 SPSS, Cary, NC, USA). All data from the study were analysed and expressed as mean \pm SD of different groups. The differences between the mean values were evaluated by one way ANOVA. $p < 0.05$ was considered significant.

RESULTS

Table 1: Oxidative status in Control, Diclofenac alone and CMSO pre-treated rats

Group	MDA (nmol/L)	CAT(U/L)	PROG (ng/ml)
A(Control)	1.18 \pm 0.20	26.49 \pm 0.27	3.89 \pm 0.50
B (100mg DIC only)	3.05 \pm 0.7*	29.98 \pm 2.70*	6.24 \pm 0.86*
C (2ml CMSO+ 100mg DIC)	2.17 \pm 0.40	23.68 \pm 0.70#	5.37 \pm 1.48
D (4ml CMSO+100mg DIC)	1.78 \pm 0.01#	20.21 \pm 1.08#	4.72 \pm 0.94#

The values are the means \pm SD. n = 6 rats in each group

*significant increase at $p < 0.05$ compared with the control (Group A)

#significant decrease at $p < 0.05$ compared with Diclofenac Alone (Group B).

On comparison of the mean values between Control and DIC alone treated groups, there was a significant ($p < 0.05$) increase in the level of MDA in DIC alone group. In contrast, there was a decrease ($p < 0.05$) in MDA as observed in group treated with high dose CMSO and DIC. CAT level was significantly ($p < 0.05$) elevated in the group treated with DIC only compared to control. However, post treatment with low and high doses of CMSO after DIC administration showed significantly ($p < 0.05$) decreased CAT level compared to group B. Furthermore, the result from the present study also showed that progesterone (PROG) level was significantly ($p < 0.05$) higher in DIC alone group B compared to group A (control). Similarly, the level of progesterone was significantly ($p < 0.05$) decreased only in group given high doses of CMSO after DIC administration.

Histology of the Ovary in Control, Diclofenac alone and CMSO pre-treated rats

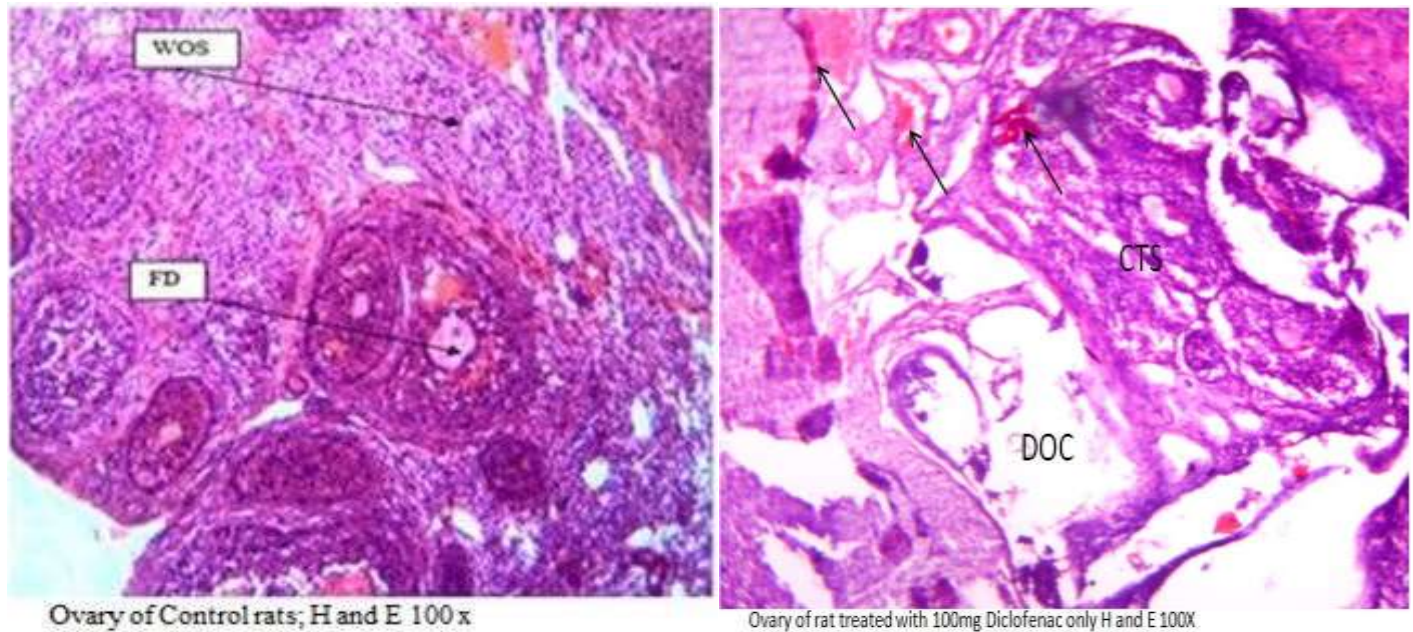
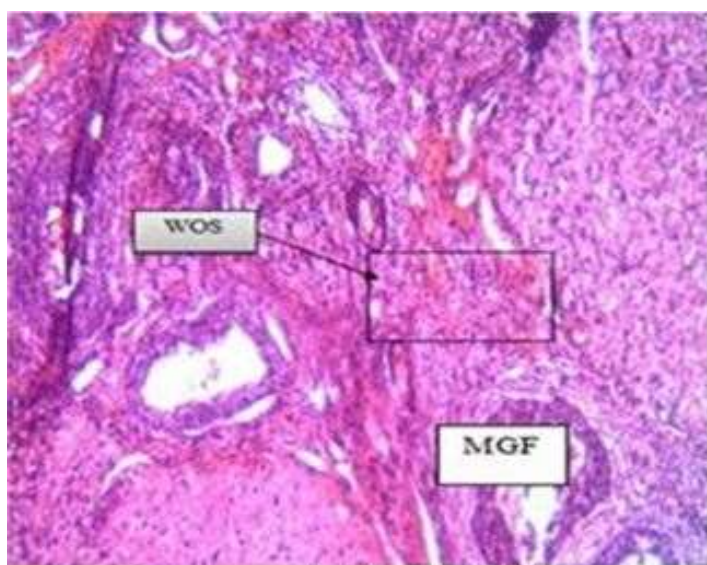
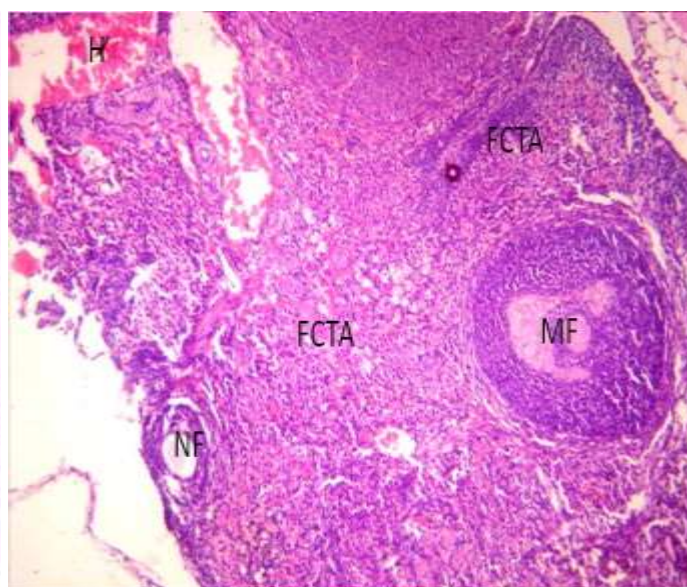


Plate 1: Micrograph showing normal architecture of the ovary in control rats with presence of various follicular development (FD) and whorl-like ovarian stroma (WOS). Contrastingly, micrograph of rats treated with 100mg DIC only showed cortical connective tissue sloughing (CTS), Developing Ovarian cyst (DOC), and variable areas of haemorrhages (black arrow). H and E, 00x



Ovary of rats pre-treated with 2ml/kg CMSO
H and E 100 x



Ovary of rat pre-treated with 4ml/kg of CMSO before Diclofenac H and E 100X

Plate 2: Micrograph sections of the ovaries in rats pre-treated with 2ml/kg CMSO followed by 100mg DIC administration shows moderately disrupted cortical tissue architecture with presence of moderately grown follicles (GF) and deranged whorled ovarian stroma (WOS) (H/E) (100x). However, ovarian sections of the rats pre-treated with 4ml/kg CMSO followed with 100mg of DIC showed better preservation of the tissues with mild Haemorrhage (H) Mature Follicle (MF), developing normal follicles (NF) within Fine Connective tissue architecture (FCTA).

DISCUSSION

The surge in MDA level following treatment with diclofenac alone corroborates earlier report that increases in MDA levels are hallmarks of oxidative stress associated with reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide (Eraslan et al., 2013). The primary reactive species is dismutated to H_2O_2 and subsequently converted to water by CATs, in a bid to mop up the free radical ions. A truncation of this process, can lead to the variable expression of CAT, an endogenous anti-oxidant involved in the cellular defence mechanism. The decreased lipid peroxidation and concomitant reduction in the expression of CAT due to supplementation of *Cucurbita maxima* seed oil can be attributed to the presence of flavonoids, phenols and other exogenous antioxidants present in CMSO which may complement the body's endogenous anti-oxidant defence the in maintaining ROS/Antioxidant status. This assertion may support to a similar report on the antioxidant capacity of *Cucurbita maxima* seed (Oyeleke et al., 2019). The decrease in CAT levels in pre-treatment groups suggests recovering from the elevated oxidative stress. Though, it may not be sufficient for prognosis when evaluated singly as its activity may vary. Our finding is in tandem with report that an increase in intracellular ROS level, hydrogen peroxide, reduces the expression of CAT (Liu et al., 2019).

Progesterone production by the ovaries helps to regulate the monthly menstrual cycle, prepare the body for conception as well as stimulate sexual desire (Montaseri et al., 2007). The increased

level of progesterone (PROG) level in diclofenac alone treated rats may initiate feedback inhibition of gonadotropin-releasing hormone (GnRH) secretion, providing the basis for the most widely-used form of contraception. Such feedback inhibition of (GnRH) prevents the mid-cycle surge of LH and ovulation. This lends credence to an earlier documented report that NSAIDs like diclofenac have been reported to affect ovulation and ovarian functions (ELAR, 2015). Implicitly, the surge in progesterone could inadvertently affect female reproduction by inhibiting embryo implantation and decidualization in line with earlier report by Liang et al. (2018). The decreased level of progesterone in high dose CMSO pre-treated rats (group D) suggests the ability of CMSO to trigger molecular signals that could relatively suppress progesterone hyper-secretion and activities associated with Hypothalamic-pituitary-gonadal axis. This finding may corroborate similar established report by Motamed et al. (2015).

Histologically, the degeneration of the ovarian tissue caused by Diclofenac therapy alone may suggest progressive atrophy of the ovarian tissue as well as a predisposition to formation of ovarian cysts and apoptosis marked by cell death and genomic DNA fragmentation provoked by oxidative stress in germ cells. The finding of this present study confirms earlier report by Devine et al. (2012) that diclofenac sodium induces variable depth of toxicity in tissues. However, the mild to moderate protection of CMSO against the ovarian associated histopathological changes buttresses the report on the efficacy of pumpkin seed oil to assuage tissue damage caused by NSAIDs as well as being a

possible therapeutic regimen for analgesic formulation with minimal side effects based on its inherent compositions (Khan et al., 2012; Fatma-Sayed 2014; Fawzy et al., 2018)

Conclusion: The present result has succinctly shown that *Cucurbita Maxima* seed oil (CMSO) is of ethnopharmacological benefit as it played a protective role against diclofenac induced ovarian cytotoxicity and oxidative stress. Further studies are recommended to isolate the phyto-constituents of CMSO for the development of adjuvants for use alongside NSAIDs in the management of pain and inflammations.

Conflict of Interest

The authors declare that there were no competing interests.

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