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Vitamin C suppresses ovarian pathophysiology in experimental polycystic ovarian syndrome

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ABSTRACT

Background: Polycystic ovary syndrome (PCOS), also known as the Stein-Leventhal syndrome is one of the most common causes of anovulation, infertility and hyperandrogenism in women, affecting between 5–10 % of women of reproductive age (12–35 years) worldwide. Despite substantial effort to define the cause of PCOS, its pathophysiology remains poorly understood. Consequently, determining the mechanisms of PCOS and the possible treatment is the major goal of medical research in endocrine and reproductive physiology.
Aim: To investigate the mechanism of ovarian metabolic changes in dehydroepiandrosterone (DHEA)-induced polycystic ovary in Wistar rats treated with vitamin C.
Methods: Twenty-eight immature female Wistar rats weighing (16–21 g) were randomly divided into four groups (n = 7/group): group I served as control and was given water, group II were injected with DHEA (6 mg/100 g in 0.2 ml corn oil subcutaneously to induce PCOS condition), group III received 150 mg/kg BW of Vitamin C orally, group IV were co-administered with 6 mg/kg BW DHEA in 0.2 ml of corn oil subcutaneously and 150 mg/kg BW of Vitamin C orally. All treatments lasted for 15 days. Twenty-four hours after the last administration, the rats were sacrificed by cervical dislocation. Blood samples and ovaries were collected for reproductive hormonal analysis, biochemical and histopathological analysis. The expressions of mRNA androgen receptor gene in the ovary were determined by real-time reverse transcriptase polymerase chain reaction. All data were analysed using one-way ANOVA.
Results: There was a significant decrease ($p < 0.05$) in the antioxidant and metabolic enzyme activity in the DHEA treated group compared with the control group. DHEA co-administration with Vitamin C showed a significant decrease in Malondialdehyde, cytokines and Estrogen and a significant increase ($p < 0.05$) in antioxidant and metabolic enzymes compared with DHEA treated group only. The histopathological evaluation demonstrates a reduction in cystic and atretic ovaries, increased expression of Bcl_2 and E-Cadherin with a reduction in *Bax* expression in the group co-administered with DHEA and Vitamin C. The DHEA group showed overexpression of mRNA Androgen Receptor gene in the ovaries compared to the control group.
Conclusion: This study shows that Vitamin C plays a protective role against DHEA-Induced Polycystic Ovary in Wistar rats via its antioxidant and anti-apoptotic mechanisms.
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1. Introduction

Infertility is the inability of a sexually active non-contracepting couple to achieve spontaneous pregnancy in one year of unprotected sexual intercourse [1]. According to Idrisa et al. [2], about 20–35 % of infertility cases are due to female factor commonly

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caused by ovulatory problems which are generally manifested by sparse or absent of menstrual periods [3,4]. Polycystic ovary syndrome (PCO) also known as the Stein-Leventhal syndrome is one of the most common causes of anovulatory infertility and hyperandrogenism in women, affecting between 5–10 % of women of reproductive age (12–35 years) worldwide [5]. Abnormalities (cyst formation, reduced ovarian reserve, reduced quality of eggs, chromosomal abnormalities, mitochondrial dysfunction, and anovulation) in the ovaries are the primary effects of this disorder caused by hyperandrogenism [6].

Dehydroepiandrosterone (DHEA) is the major secretory steroidal product of the adrenal gland and the ovary with serum concentration 20 times higher than that of other steroid hormones [7]. It has been reported that hyperandrogenization of BALB/c mice with DHEA prevents ovulation by increasing ovarian oxidative stress and altering the endocrine or immunological system leading to PCO [8]. The markers of oxidative stress or inflammation are correlated with increased androgen level in women with PCOS [9]. This syndrome involves frequent abnormalities of lipid or glucose metabolism that emerge as factors in the pathogenesis of the syndrome [10].

Oxidative stress seems to be involved in altered steroidogenesis in the ovaries, thus contributing to increased androgen production, disturbed follicular development and apoptosis, ultimately leading to infertility [11,12]. Apoptosis is considered an important feature of PCOS and has been suggested to participate in the pathogenesis and development of the syndrome [13]. Despite substantial effort to define the cause of PCOS, its pathophysiology remains poorly understood. Consequently, determining the mechanisms that cause PCOS and the possible treatment is a major goal of medical research in endocrine and reproductive physiology.

Vitamin C has been demonstrated to be an effective antioxidant. It acts both directly, by reaction with aqueous peroxyl radicals, and indirectly, by restoring the antioxidant properties of fat-soluble vitamin E. The overall consequence of these antioxidant activities is the beneficial control of lipid peroxidation of cellular membranes including those surrounding as well as within intracellular organelles. Intracellular free radical attack on non-lipid nuclear material may also be diminished [14]. Vitamin C plays an important role in the regulation of the menstrual cycle and ovarian function. Ascorbic acid excretion is increased and declines immediately prior to ovulation, and then immediately increases again just after temperature rises post-ovulation. This reflects uptake of ascorbic acid in the pre-ovulatory ovary, which then facilitates proper ovulation. These ascorbic acid levels are stimulatory to the hormones progesterone and oxytocin and have been found in high concentrations in the corpus luteum [15]. High levels of ascorbic acid present in the ovaries may be responsible for collagen synthesis, which is required for follicle and corpus luteum growth, as well as repair of the ovary post-ovulation.

Problems with this function may contribute to the development of ovarian cyst [16] Available reports on the effects of vitamin C on ovarian biology, polycystic ovarian syndrome, and pre-implantation embryonic development are still very scanty. Therefore, this study aimed at investigating the role of vitamin C in suppressing ovarian pathophysiology in experimental polycystic ovary syndrome. Hence, we hypothesized that co-treatment with vitamin C would ameliorate the ovarian lesions in antioxidant pathways caused by DHEA in Wistar rats.

2. Materials and methods

Twenty-eight (28) pre-pubertal female Wistar rats of 21 days old weighing 16–21 g were obtained from the Department of Human Physiology, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan and housed in cages in a well-ventilated animal house of Bingham University, Karu, Nasarawa State, Nigeria. They were provided with rat pellets and water *ad libitum*. The ethical approval on animal act right was obtained from the Institutional Animal Care Committee of the same Institution with assigned number BHU-ACURE/18/0124. All the experimental procedures were done following the experimental guidelines of the Institutional Animal Ethics Committee (IAEC) of Bingham University, Karu, Nasarawa State, Nigeria.

2.1. Animal treatments and tissue collection

Twenty eight immature female Wistar rats (21 days old) weighing 16–21 g were randomly divided into four groups (n = 7/group): The rats in group I served as control and were given distilled water daily, group II were injected with DHEA (6 mg/100 g body weight in 0.2 ml corn oil subcutaneously daily to induce PCOS condition) [17], group III were administered with Vitamin C (150 mg/kg body weight orally daily) and group IV were injected with DHEA (6 mg/100 g body weight in 0.2 ml corn oil subcutaneously) and 150 mg/kg body weight vitamin C orally respectively. All treatments lasted for 15 days [18]. Twenty-four hours after the last day of administration, blood samples from all the rats were collected via the retro-orbital venous sinus [19] and serum samples were obtained for the determination of female sex hormones (Progesterone and estrogen). Rats were then sacrificed by cervical dislocation. The ovaries were collected and cleared of adherent connective fat tissue for further biochemical study. One ovary from each rat was rapidly submerged in RNA later and stored at 4°C until used for the determination of mRNA androgen receptor gene expression using real-time reverse transcriptase polymerase chain reaction (qPCR) and one ovary was fixed in Bouin's fluid and processed for histopathological analysis.

2.2. Oxidative stress-related parameters

2.2.1. Lipid peroxidation assay

Malondialdehyde (MDA) levels were estimated by the method described by Dahle et al [20]. Thiobarbituric acid reactive substances – TBARS was measured as an indicator of lipid peroxidation and ROS by extension. Serum samples were placed in a microcentrifuge tube and incubated with thiobarbituric acid (TBA). Following incubation, the samples were centrifuged (2000 rpm, 10 min) and the absorbance of the pink clear supernatant was measured at 532 nm in duplicate samples. Malondialdehyde bis-(dimethyl acetal) was used as the external standard. Thiobarbituric acid reactive substances were expressed in terms of nanomoles of MDA/gram of wet tissue. Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation [21].

2.2.2. Determination of tissue superoxide dismutase activity

The activity was expressed as unit/mg of protein. The level of SOD activity was determined by the method described by Misra and Fridovich [22]. The ability of SOD to inhibit the autoxidation of epinephrine at pH 10.2 makes this reaction the basis for a simple assay for superoxide dismutase. The superoxide ($O2^{\bullet}-$) radical generated by the xanthine oxidase reaction causes the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per introduced $O2^{\bullet}-$ increases.

2.2.3. Determination of tissue catalase activity

Catalase activity was determined according to the method described by Sinha [23]. This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2 with the formation of perchromic

acid as an unstable intermediate. The chromic acetate then produced is measured by colorimetric analysis at 570–610 nm. Since dichromate has no absorbance in this region, the presence of the compound in the assay mixture does not interfere at all with the colorimetric determination of chromic acetate. The catalase preparation is allowed to split H_2O_2 for different periods of time. The reaction is stopped at a particular time with the addition of a dichromate/acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate by colorimetric analysis after heating the reaction mixture.

2.2.4. Determination of tissue glutathione-S-transferase activity

Glutathione-S-transferase (GST) activity was determined according to the method described by Habig et al. [24]. The method is based on the principle that glutathione-S-transferase demonstrates a relatively high level of activity in the presence of 1-chloro-2,4-dinitrobenzene (CDNB), the substrate used in the assay to measure GST activity. When CDNB is conjugated with reduced glutathione, the absorption maximum shifts to a longer wavelength. The absorption increase at the new wavelength of 340 nm provides a direct measurement of the enzymatic reaction.

2.2.5. Reduced glutathione assay (GSH)

Reduced glutathione was estimated by the method of Jollow et al. [25] by using 1, 2-dithio-bis nitro benzoic acid (DTNB) as substrate. The yellow color developed was read immediately at 412 nm and expressed as μmol GSH/g tissue.

2.3. Total protein (Biuret reagent)

Protein content of the tissue samples was determined using the method described by Lowry et al. [26].

2.4. Cytokines

2.4.1. Test principle tissue vascular endothelial growth factor (VEGF)

The ELISA kit used was Sandwich-ELISA principle. The micro ELISA plate provided in this kit was pre-coated with an antibody specific to vascular endothelial growth factor (VEGF). Standards or samples were added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for VEGF and Avidin-Horseradish Peroxidase (HRP) conjugate were added successively to each microplate well and incubated. Free components were washed away. The substrate solution was added to each well. Only those wells that contain VEGF, biotinylated detection antibody and Avidin-HRP conjugate appeared blue in color. The enzyme-substrate reaction was terminated by the addition of stop solution and the color turned yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of VEGF. The concentration of VEGF was calculated in the samples by comparing the OD of the samples to the standard curve.

2.4.2. Test principle tissue tumor necrosis factor-alpha (TNF-α)

The Enzyme-Linked Immunosorbent Assay (ELISA) kit was used in the study. TNF-α was added to the wells pre-coated with TNF-α monoclonal antibody. After incubation, a biotin-conjugated anti-Rat TNF-α antibody was added and binds to Rat TNF-α. After incubation unbound biotin-conjugated anti-Rat TNF-α antibody was washed away during the washing step. Streptavidin-HRP was added and binds to the biotin-conjugated anti-Rat TNF-α antibody. After incubation unbound Streptavidin-HRP was washed away during the washing step. Substrate solution was then added and color develops in proportion to the amount of rat TNF-α. The reaction was terminated by addition of acidic stop solution and absorbance was measured at 450 nm.

2.4.3. Estimation of serum progesterone and estrogen levels

The serum samples obtained were analyzed to determine the concentrations of progesterone and estrogen hormone. The analysis was carried out via tube-based enzyme immunoassay (EIA) method. The protocol used in hormone testing followed the method described by the kit manufacturers (Immunometrics Limited UK) and met the WHO research program standards for reproductive studies.

2.5. Determination of ovarian proton pump (ATPase) activity

Na^+/K^+-ATPase, Ca^{2+} ATPase, and H^+ ATPase activities were analysed based on a modification of the method published by Evans [27]. Exactly, 1 mM Na_2HP0_4 (142 mg of disodium hydrogen phosphate (BDA Chemicals Co, Ltd, England) in a 100 ml of water) was used as the standard curve for the determination of the inorganic phosphate liberated. The procedure was adopted from Stewart [19], which is based on a colour reaction developed using 1.25% NH_4 molybdate (6.25 g of ammonium molybdate (Hopkins and Williams Ltd; England) was dissolved in 500 ml of 6.5% sulphuric acid (BDH Chemicals Ltd, England))and 9% Ascorbic acid (22.5 g of L-ascorbic acid (Sigma Chemical Co; USA) was dissolved in 250 ml of distilled water. Protocol for inorganic phosphate determination was according to the procedure of Fiske and Subbarow [5] (Table 1).

2.6. Determination of Na^+/K^+, Ca^{2+} and H^+ ATPase activity in the ovarian homogenate Expression of enzyme activity (μmole pi / mg protein / hour x 10^{-3})

Approximately, 0.5 ml of each of 0.35 M of sodium chloride, 17.5 potassium chloride, for Na^+/K^+ ATPase, 0.5 ml of each of 17.5 mM calcium Chloride for Ca^{2+} ATPase and 0.5 ml of each of 17.5 mM potassium chloride for H^+ ATPase, 21.0 mM magnesium chloride, 10 mM of Tris HCl at pH 7.4 mM and 8.0 mM Disodium ATP are mixed together in a test tube. 0.2 ml of tissue homogenate was added to it and incubated at 37 °C for 60 min. The reaction was terminated by the addition of 0.8 ml of ice-cold 10% (w/v) trichloroacetic acid (TCA), it was allowed to stand for 20 min at 4 °C. Centrifuge at 4000 rpm for 5 min. 1 ml of supernatant was then added to 1 ml of 25% ascorbic acid, kept at room temperature for 20 min and the absorbance was measured at 725 nm using spectrophotometer according to the methods of Bonting [28,29] and Hjerken and Pan [30] for Na^+/K^+ and Ca^{2+} ATPases, respectively and the enzymes were assayed by the modified method of Evans [31]. For H^+ ATPase, 1 ml of the supernatant, add 1 ml of 1.25% Ammonium molybdate and wait for 10 min. Then 1 ml of 9% ascorbic acid was added, kept at room temperature for 20 min and the absorbance was measured at 725 nm using spectrophotometer according to the method of Ohinishi et al. [32] and the enzyme was similarly analysed by the modified method of Evans [31].

2.7. Histological techniques

Histological examination was carried out on the tissues fixed in Bouin's fluid. Tissue blocks were sectioned for routine Hematoxylin and Eosin (H&E). The fixed organs were cut in slabs of about 0.5 cm thick transversely and transferred to 70% alcohol for dehydration. The tissues were passed through 90% and absolute alcohol and xylene for different durations before they were transferred into two changes of molten paraffin wax for 1 h each in an oven at 65 °C for infiltration. They were subsequently embedded and serial sections using a rotary microtome at six microns (6 μ). The tissues were transferred onto albumenized slides and allowed to dry on

Table 1
The procedure for the standard curve for the determination of the inorganic phosphate liberated.

Test tubes in duplicates	1	2	3	4	5	6	7	8	9
1 mM Na_2HP0_4	–	0.2	0.4	0.6	0.8	1.0	1.2	2.0	3.0
Distilled water (ml)	10.0	9.8	9.6	9.4	9.2	9.0	8.8	8.0	7.0
1.25% Ammonium molybdate (ml)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Ascorbic acid (ml)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

a hot plate for 2 min. The slides were dewaxed with xylene and passed through absolute alcohol (2 changes); 70% alcohol, 50% alcohol and then to water for 5 min. The slides were then stained with hematoxylin and eosin.

2.8. Immunohistochemistry (Bax, BCl_2, and E-Cadherin)

Samples for immunohistochemical studies were fixed in 10% formalin, and then after dehydration and embedding in paraffin cut into 7 μm sections. To identify Bax, BCl2, and E-Cadherin proteins, preparations from the groups were used. For each preparation, a negative control was performed (a slide without primary antibody). The proteins expression level was evaluated with a standard three-step immunohistochemical procedure. Rabbit Bax, BCl2 and E-Cadherin antibodies were used as a primary antibody. Then biotinylated secondary antibody was added, and then horse-radish peroxidase conjugated with streptavidin. Since streptavidin has a great affinity to biotin, it binds to the place where primary antibody coated the background, and after adding a chromogen (DAB) a reddish colour appears.

2.9. mRNA Androgen receptor gene expression using qPCR

2.9.1. RNA extraction

The Total RNA Mini Kit was designed specifically for purifying total RNA from a variety of animal tissue. Samples were efficiently homogenized in a microcentrifuge tube using the provided micropestle. Detergents and chaotropic salt were used to lyse cells and on-column DNase treatment was used to remove contamination of genomic DNA. RNA in the chaotropic salt is bound by the glass fiber matrix of the spin column. Once any contaminants have been removed, using the Wash Buffer (containing ethanol), the purified total RNA is eluted by RNase-free Water.

2.9.2. Agarose gel electrophoresis

The extracted RNA was checked on 1% agarose gel electrophoresis. The gels were stained with Ethidium Bromide and visualized under Bluelight transilluminator. For a 10 cm x 10 cm mini-gel cast, 1% agarose gel was prepared by dissolving 0.4 g of agarose in 40 ml of 1x TAE buffer. The mixture of agarose and buffer was swirled gently to ensure complete dissolution. The colloidal solution formed was heated at medium heat in the microwave oven for 1–3 min or until a clear solution was obtained. The gel was allowed to cool to about 50 °C (gel should not solidify) under a running tap. The precaution was taken to prevent water from the running tap from splashing into the gel. Ethidium Bromide was added to a concentration of 0.5 μg/ml (2 μl stock in 40 ml) and mixed by swirling till no trace of the stain was detected. The gel was poured into the gel tray set with combs and allowed to solidify. This normally takes about twenty min. After the gel was set, the casts were removed and the gel tray (with the solidified gel) set in the gel tank, submerged in the running buffer (1x TAE). Afterward, the gel comb was gently removed from the submerged gel and the amplicons were loaded in the wells created by the comb. The genomic RNA extracted from each of the samples was mixed with a 6x loading dye in the ratio of 5 μl of the sample/ amplicon to 1 μl of loading buffer. This was combined individually and loaded into the wells.

2.9.3. cDNA synthesis

Exactly 1 μg of each of the extracted RNA samples was converted to cDNA using the Bioline SensiFAST cDNA synthesis kit (according to manufacturer's protocol). The reaction contains 1 μl of Reverse Transcriptase and 4 μl of 5x TransAmp Buffer. Nuclease-Free Water was added to make reaction volume up to 20 μl. The thermocycling conditions were as follows: Annealing at 25 °C for 10 min, Reverse Transcription at 42 °C for 15 min, an additional 48 °C for 15 min because of the highly structured RNA and lastly, inactivation of the enzyme at 85 °C for 5 min.

2.9.4. Real-time polymerase chain reaction

The synthesized cDNA was amplified using the Bioer LineGene 9600 Real-Time PCR machine. This was done in 10 μl reactions consisting of 2 μl of the template, 2 μl of the Solis Biodyne 5x qPCR mix, 0.25 μl each of the forward and reverse primers and 5.5 μl of nuclease-free water. This process was carried out for all the samples and a No Template Control reaction was made in which water was substituted for the template. The qPCR conditions were as follows: Initial activation at 95 °C for 12 min, Denaturation at 95 °C for 15 s, annealing at 65 °C for 20 s and Elongation at 72 °C for 20 s. Same cycling conditions were used for the housekeeping gene βActin but the annealing temperature was changed to 64 °C. The electrophoresis was run at 75 V for 1 h, after which it was viewed and photographed under blue light transilluminator. Solis biodyne 100 bp DNA ladder was run alongside the extracted RNA samples.

2.10. Data analysis

The results were expressed as Mean ± standard error of mean (SEM), and subjected to statistical analysis using the ANOVA GraphPad Prism software version 6, Tukey post-hoc analysis for data analysis. P value <0.05 was considered significant and $p < 0.01$ indicates highly significant differences while $p > 0.05$ indicates non-significant differences.

3. Results

3.1. Effects of oral administration of 150 mg/kg BW Vitamin C on ovarian malondialdehyde level in DHEA-Induced polycystic ovary in Wistar rats

The results obtained showed that the group co-administered with Vitamin C and DHEA caused a significant decrease ($p < 0.05$) in MDA level when compared with the DHEA group alone. Dehydroepiandrosterone treated group showed a significant increase ($p < 0.05$) in malondialdehyde level when compared with the control group (Fig. 1).

3.2. Effects of oral administration of 150 mg/kg BW Vitamin C on ovarian antioxidant activity in DHEA-Induced polycystic ovary in Wistar rats

The results showed that DHEA group caused a significant decrease ($p < 0.05$) in the level of antioxidant activity when compared with control in Wistar rats. The group co-administered with

Table 2
Effects of oral administration of 150 mg/kg vitamin C on antioxidant enzymes in Dehydroepiandrosterone-Induced polycystic ovary in Wistar Rats.

Groups	SOD U/ml/mgprotein	CAT μmole/min/mgprotein	GST μmole/min/mgprotein	GSH mg/ml/mgprotein
I	1.63 ± 0.03	103.3 ± 3.19	0.08 ± 0.01	7.50 ± 0.52
II	1.52 ± 0.04	92.9 ± 0.04^{+}	0.06 ± 0.00^{+}	6.09 ± 0.48
III	1.78 ± 0.06	116.5 ± 3.74	0.08 ± 0.01	8.35 ± 0.67
IV	1.67 ± 0.04	107.5 ± 2.31*	0.07 ± 0.00	7.02 ± 0.68

Values are represented in mean ± SEM, n = 7/group.
$^{+}$ $p < 0.05$ when compared with control value.
* $P < 0.05$ when compared with DHEA treated group, SOD (Superoxide Dismutase), CAT (Catalase), GST (Glutathione-S-Transferase), GSH (reduced glutathione).

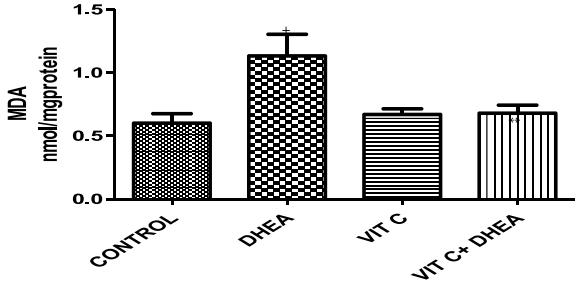


Fig. 1. Effects of oral administration of 150 mg/kg Vitamin C on malondialdehyde level in Dehydroepiandrosterone-Induced polycystic ovary in Wistar Rats.
Values are represented in mean ± SEM, n = 7/group, + $p < 0.05$ when compared with the control value.
*$P < 0.05$, when compared with DHEA, treated group, **$p < 0.01$, when compared with DHEA, treated group.

Table 3
Effects of oral administration of 150 mg/kg vitamin C on ATPase enzymes activity in Dehydroepiandrosterone-Induced polycystic ovary in Wistar Rats.

Groups	Na^{+}/K^{+}ATPase (Piμmol/mgprotein/hr/10^{-3})	Ca^{2+}ATPase	H^{+}ATPase
I	0.49 ± 0.04	4.72 ± 0.19	0.44 ± 0.00
II	0.41 ± 0.01	4.02 ± 0.06^{+}	0.42 ± 0.01^{+}
III	0.49 ± 0.01	4.71 ± 0.36	0.48 ± 0.01
IV	0.50 ± 0.02*	4.58 ± 0.21*	0.48 ± 0.02*

Values are represented in mean ± SEM, n = 7/group.
$^{+}$ $p < 0.05$ when compared with control value.
* $P < 0.05$ when compared with DHEA treated group.

Vitamin C and DHEA showed an increase in the level of antioxidant as compared with DHEA alone treated group (Table 2).

3.3. Effects of oral administration of 150 mg/kg BW Vitamin C on serum estrogen and progesterone concentrations in DHEA-Induced polycystic ovary in Wistar rats

The results obtained showed that DHEA treated group caused a significant decrease ($p < 0.05$) in the level of progesterone and significant increase ($p < 0.05$) in estrogen concentrations as compared with control. The group co-administered with Vitamin C and DHEA caused a significant increase ($p < 0.05$) in the level of progesterone when compared with the DHEA group (Fig. 2).

3.4. Effects of oral administration of 150 mg/kg BW Vitamin C on ovarian proton pump ATPase enzymes activity (Na^{+}/ K^{+}, $Ca^{2+}$and H^{+} ATPase) activity in DHEA-Induced polycystic ovary in Wistar rats

The results obtained showed that the group co-administrated with Vitamin C and DHEA caused a significant increase ($p < 0.05$) in proton pump enzymes activity when compared with DHEA alone. Also, DHEA treated group showed a reduction in the activity of these enzymes as compared with the control group (Table 3).

3.5. Effects of oral administration of 150 mg/kg BW Vitamin C on ovarian inflammatory cytokines (Tumor necrosis Factor- alpha and Vascular Endothelial Growth Factor) concentrations in DHEA-Induced polycystic ovary in Wistar rats

The results obtained showed that DHEA treated group caused a significant increase ($p < 0.05$) in TNF-α and VEGF concentrations when compared with the control group (Fig. 3). The group co-administered with Vitamin C and DHEA caused a significant decrease ($p < 0.05$) inflammatory cytokines level when compared with DHEA alone

3.6. Effects of oral administration of 150 mg/kg BW Vitamin C on the granulosa cell mRNA Androgen receptor gene expression in DHEA-Induced polycystic ovary in Wistar rats

The results obtained showed that DHEA treated group showed a significant expression ($p < 0.05$) in mRNA androgen receptor gene in the ovarian granulosa cells when compared to the control group. The group co-administered with Vitamin C and DHEA caused a significant decrease ($p < 0.05$) in mRNA androgen receptor gene expression as compared to DHEA alone (Fig. 4).

4. Discussion

There was a significant decrease in the antioxidant and metabolic enzyme activity in the DHEA treated group as compared with the control group. DHEA co-administration with Vitamin C showed a significant decrease in malondialdehyde, cytokines, and estrogen and a significant increase in antioxidant and metabolic enzymes when compared with DHEA treated group only. The histopathological evaluation demonstrates a reduction in cystic and atretic ovaries, increased expression of Bcl_2 and E-Cadherin with reduction in *Bax* expression in the group co-administered with DHEA and Vitamin C (Plate A–P). The DHEA group showed overexpression of mRNA Androgen Receptor gene in the ovaries compared to the control group (Figs. 4, 5 and 6).

Polycystic Ovarian Syndrome has been linked with some malignant lesions such as endometrial cancer, breast cancer and ovarian cancer [33]. Oxidative stress altered in PCOS is known to play a pivotal role in cancer pathogenesis [34]. Reactive Oxygen Species could cause genetic changes by attacking DNA, leading to DNA damage and mutations in protooncogenes and tumor suppressor genes by hijacking cell proliferation out of control when the DNA repair mechanism has been disrupted [35]. On the other hand, oxidative stress could also cause epigenetic changes as well by DNA methylation, silencing tumor suppressor genes [36]. Low concentration of plasma vitamin C is known to occur in several conditions of increased oxidative stress, such as cancer, diabetes mellitus, cataract, HIV infection and cigarette smoking [37]. In addition, an early report showed that daily supplementation with vitamin C at high doses (grams) increased the survival time of terminal cancer patients [38] and it was suggested that vitamin C could have important anticancer properties [39]. Indeed, vitamin C inhibits

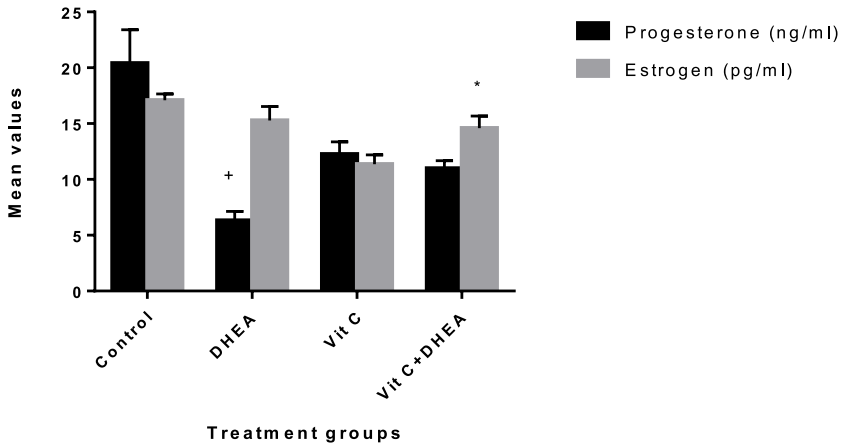


Fig. 2. Effects of oral administration of 150 mg/kg Vitamin C on progesterone and estrogen level in Dehydroepiandrosterone-Induced polycystic ovary in Wistar Rats. Values are represented in mean ± SEM, n = 7/group, + $p < 0.05$ when compared with control value.
*$P < 0.05$ when compared with DHEA treated group.

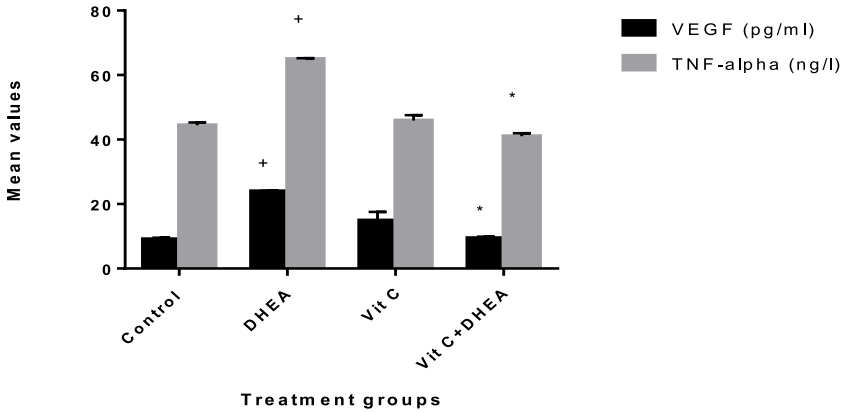


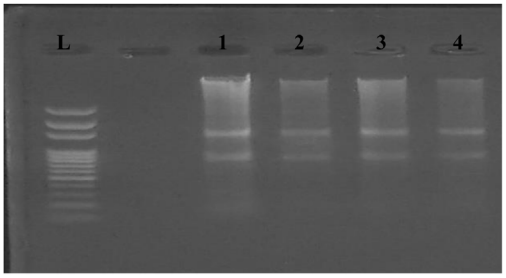
Fig. 3. Effects of oral administration of 150 mg/kg Vitamin C on VEGF and TNF-α level in Dehydroepiandrosterone-Induced polycystic ovary in Wistar Rats. Values are represented in mean ± SEM, n = 7/group, + $p < 0.05$ when compared with control value.
*$P < 0.05$ when compared with DHEA treated group.

the growth of many tumor cell lines [40] and potentiates the cytotoxicity of radio-sensitizing drugs. There are also several reports showing that cancer cell lines are more sensitive to vitamin C than their non-malignant counterparts [41]. Regarding cancer prevention, several epidemiological studies have linked the consumption of a diet rich in fruit and vegetables (antioxidants) with a lower incidence of many types of cancer [42–44]. One of the most important mechanisms and the sequence of events by which free radicals interfere with the cellular functions seem to be the lipid peroxidation leading eventually the cell death. To protect this cellular death from reactive oxygen species, living organisms have developed an antioxidant line of defense systems [45].

The results obtained showed that DHEA treated group caused a significant increase in malondialdehyde level as compared to control group. This could be due to increased generation of free radicals causing lipid peroxidation [46]. The group co-administered with Vitamin C and DHEA showed a significant decrease in malondialdehyde level compared to DHEA treated group as shown in (Fig. 1). Dehydroepiandrosterone treated group showed a significant decrease in the antioxidant enzymes activity when compared with the control group. The group co-administered with Vitamin C and DHEA showed a significant increase in antioxidant enzyme activity compared to the DHEA group as shown in Table 2.

Inflammatory markers such as Tumor Necrosis Factor (TNF-α), Interleukin -6 (IL-6) have been shown to increase in women with PCOS [47]. It is accepted that there is a tight link of oxidative stress and inflammation and it is hard to distinguish inflammation from oxidative stress absolutely [48]. Reactive oxygen species could induce releasing inflammatory factors and inflammatory response, via activating the associated signaling pathways of nuclear factor –Kappa B (NF—KB) activated protein 1 (AP-1) and hypoxia-inducible factor-1 (HIF-1) [49]. Dehydroepiandrosterone treated group showed a significant increase in TNF-α and Vascular Endothelial Growth Factor (VEGF) concentrations compared with control group. This result is in accordance with the increase in ovarian VEGF concentrations observed in PCOS patients, validating the DHEA model used in this study to determine changes in angiogenesis in PCOS [50]. The group co-administered with Vitamin C and DHEA caused a significant decrease in TNF-α and VEGF concentrations compared to DHEA group as shown in Fig. 3.

Exposure to unopposed estrogen in the absence of progesterone is known to induce be induced by anovulation, which is also regarded as a major factor causing hyperplasia and cancer formation in polycystic Ovary [51]. Estrogen could bind to its nuclear receptor, stimulating secretions of various growth factors such as Insulin Growth Factor, Epidermal growth factor and Vascular Endothelial Growth Factor activating Extracellular signal-regulated kinases (ERK) signaling pathway to promote endothelial and ovarian proliferation and even cancer formation [52]. In addition, metabolites of estrogen also could be the inducers of ovarian and endometrial cancer by binding to DNA, causing further DNA damage associated with oxidative stress. Under oxidative stress,



L=Ladder

Gel Image for Total RNA

List of primers and probe sequences

AR	
RNAR F RNAR R	ATGCTGGGCCTGTAGCCCCCT CAGGCAGGTCTTCTGGGGTGGG
B-Actin	
RNACTB F RNACTB R	CCTCCGTCGCCGGTCCACACC TCTTGCTCTGGGCCTCGTCGC

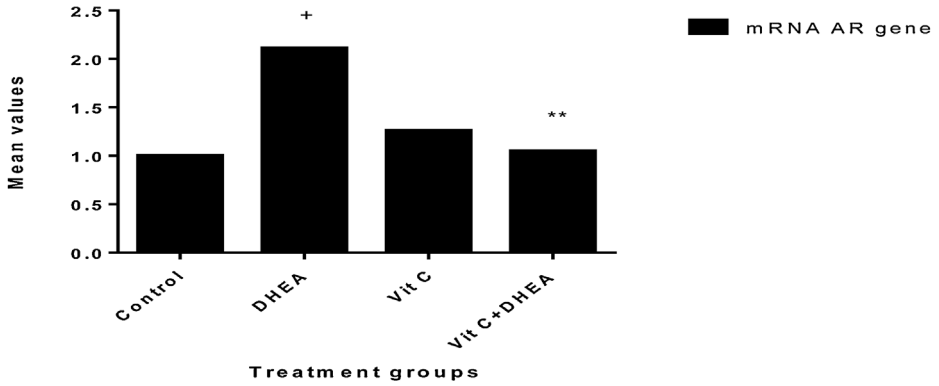


Fig. 4. mRNA androgen receptor gene fold change values (2÷ddCt) for Vitamin C and DHEA treatment.
Values are represented in mean ± SEM, n = 7/group, + p < 0.05 when compared with control value.
*P < 0.05 when compared with DHEA treated group, **p < 0.01 when compared with DHEA treated group.

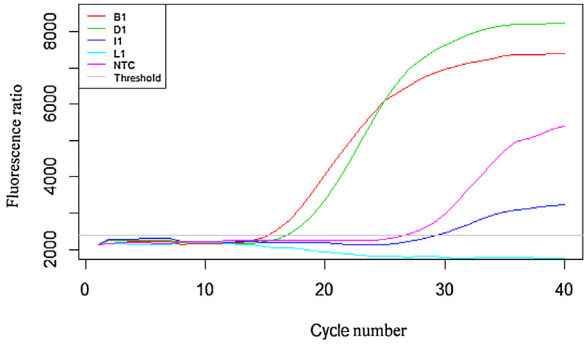


Fig. 5. Amplification curve for Androgen receptor treated with Vitamin C.
Keys: B1: control; D1:DHEA; I1: vitamin C; L1: vitamin C + DHEA; NTC: no template control.

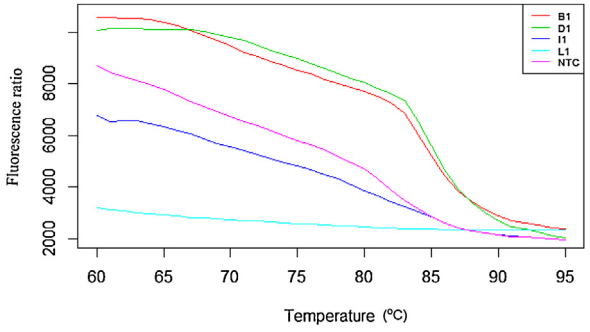


Fig. 6. Melt Curve for Androgen receptor treated with Vitamin C.
Keys: B1: control; D1:DHEA; I1: vitamin C; L1: vitamin C + DHEA; NTC: no template control.

estrogen intermediate metabolites including 2-hydroxyl estrone (2−OHEI), 4-hydroxyl etrone (4−OHEI) and 16 α -hydroxyl estrone (16α−OHEI) could not be methylated and eliminated from the body would then be oxidized to semiquinones compounds and quinones compounds [52]. The two abnormal types of metabolites with electron affinity bind to a nucleophilic group of DNA

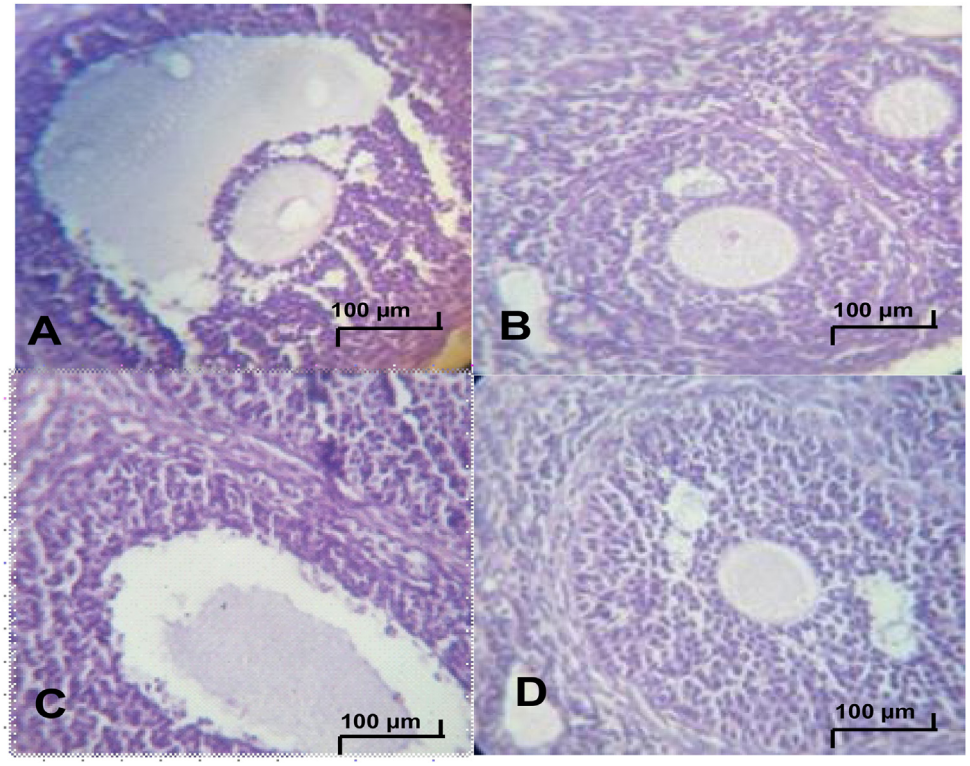


Plate 1. A – D represents histological sections of ovaries from dehydroepiandrosterone (DHEA) exposed rats and control following 15 days treatment using hematoxylin and eosin stain (H&E) X40 magnification.
A. Photomicrograph section of ovary from control group showing normal follicle of different stages with well-organized surface epithelium without cystic follicle. The stromal cells of the ovarian follicles and corpus luteum are also well developed.
B. Photomicrograph section of ovary from DHEA exposed rats showing an increasing number of cystic follicles with a thicker theca cell layer and a marked higher level of collagen especially in region around the follicle and there is absence of corpora lutea.
C. Photomicrography section of ovary from Vitamin C exposed rats showing normal histo- architecture of the ovary. The interstitial tissues are also normal.
D. Photomicrography section of ovary from Vitamin C and DHEA exposed rats showing a decrease in number of cystic follicles with a thinner theca cell layer and a reduced level of collagen especially in region around the follicle.
PLATES E – H represents Immunohistochemistry of Bax Apoptotic protein staining of ovaries from Dehydroepiandrosterone (DHEA) treated rats and control following 15 days treatment. × 40 magnifications.
E. Photomicrograph section of ovary from control group showing normal preantral follicles (PF), granulosa cells (GC) and theca cells (TC). Bax expression is mainly localized in granulosa cells of antral follicles.
F. Photomicrograph section of ovary from DHEA exposed rats showing an increasing number of cystic follicles (CY), presences of atretic follicle (AT) and Bax expression is greater in preantral and antral follicles.
G. Photomicrography section of ovary from Vitamin C exposed rats showing normal preantral, antral follicle and stroma (ST). The granulosa cells and Oocytes (Oo) are also normal.
H. Photomicrography section of ovary from Vitamin C and DHEA exposed rats showing a decrease in amount of cystic follicles (CY). Bax expression is decreased in preantral and antral follicles (AF).
PLATES I–L represents Immunohistochemistry of Bcl-2 Anti-apoptotic protein staining of ovaries from Dehydroepiandrosterone (DHEA) treated rats and control following 15 days treatment. × 40 magnifications.
I. Photomicrograph section of ovary from control group showing normal preantral follicles (PF), granulosa cells (GC) and theca cells (TC) with Bcl-2 expressions are mainly localized in granulosa cells of antral follicles.
J. Photomicrograph section of ovary from DHEA treated rats showing an increasing amount of cystic follicles. Bcl-2 protein was either absent in preantral follicles or weakly expressed in antral follicles.
K.Photomicrography section of ovary from Vitamin C treated rats showing normal preantral and antral follicle with Bcl-2 expression mainly localized in granulosa cells of antral follicles.
L. Photomicrography section of ovary from Vitamin C and DHEA treated rats showing a decrease in amount of cystic follicles. Bcl-2 expression was decreased in preantral and antral follicles (AF).
PLATES M – P represents Immunohistochemistry of E- Cadherin (adhesion molecule) staining of ovaries from Dehydroepiandrosterone (DHEA) treated rats and control following 15 days treatment. × 40 magnifications.
M. Photomicrograph section of ovary from control group showing normal expression of E-cadherin which was largely confined to areas of the interstitium, theca and surface epithelium.
N. Photomicrograph section of ovary from DHEA exposed rats showing an increasing amount of cystic follicles (CY). Expression of E-Cadherin which was largely confined to areas of the stroma indicates the reduction in the growth of the follicle.
O. Photomicrography section of ovary from Vitamin C exposed rats showing normal preantral and antral follicle. The granulosa cells are also normal with well-expressed E-Cadherin indicating the growth and development of the follicles.
P. Photomicrography section of ovary from Vitamin C and DHEA treated rats showing a decrease in quantity of cystic follicles. Expression of E-Cadherin was decreased in preatral and antral follicle, but well expressed in the granulosa cells.

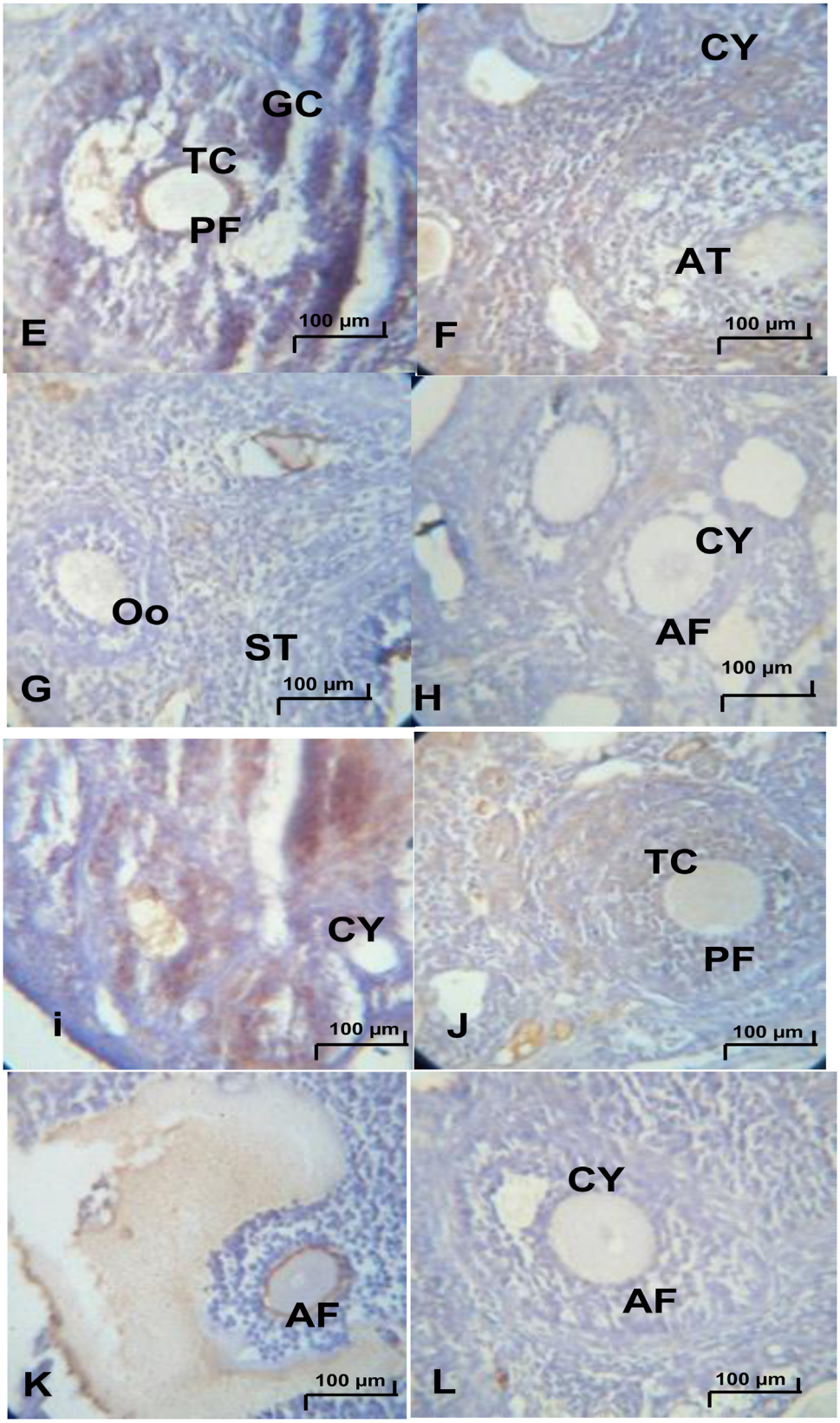


Plate 1. (*Continued*)

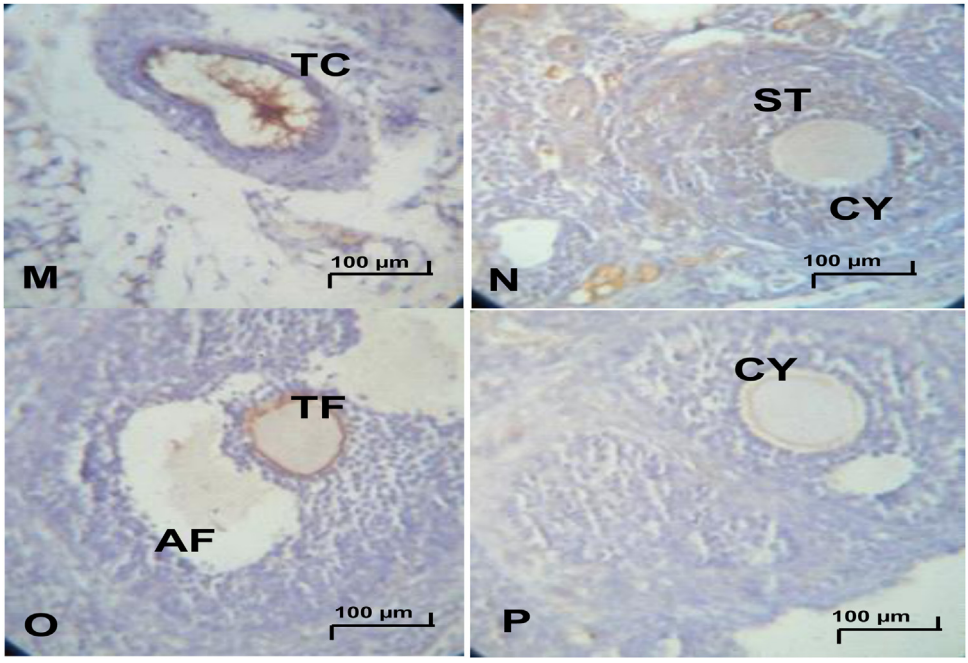


Plate 1. (*Continued*)

by covalent bond, causing DNA mutation and further leading to ovarian and endometrial cancer pathogenesis. Dehydroepiandrosterone treated group showed a significant decrease in progesterone and a significant increase in estrogen concentrations compared to the control group while the group co-administered with Vitamin C and DHEA caused an increase in progesterone level compared to the Dehydroepiandrosterone treated group as shown in Fig. 2.

It is known that DNA damage and methylation induced by oxidative stress (OS) play key roles in the early stage of tumor pathogenesis and tumor conversion by activating protooncogene and silencing antioncogene [53]. Therefore, abnormal oxidative stress in PCOS could cause genetic instability and raise the risk of cancer. Oxidative stress has been demonstrated to be significantly associated with inflammation, obesity, and hyperandrogenemia in PCOS patients, which are the common characteristics and potential inducer ovarian dysfunction. Results from several studies have implicated decreased proton pump (ATPases) activity in female reproductive dysfunction. Oxidative stress has been suggested as a contributory factor to impaired ATPase activity [54,55] ATPase has been shown to be very susceptible to free radicals and membrane lipid peroxidation and inflammation. Lipid peroxidation has been shown to alter Na^+/K^+-ATPase, calcium ATPase and magnesium ATPase functions by modification at specific active sites in a selective manner [56]. Depletion of glutathione and other protective antioxidants by reactive oxygen species may greatly contribute to increasing the levels of reactive species, which may also account for the impaired activity of Na^+/K^+, Ca^{2+} and H^+ -ATPase [57,58]. The group co-administered with Vitamin C and DHEA showed a significant increase in Na^+/K^+, Ca^{2+}, H^+ ATPase activity compared to the DHEA group. Proton pumps maintain trans-membrane gradients for the ions and produce a convenient driving force for the secondary transport of metabolic substrates such as amino acids and glucose. ATPases are responsible for proper cellular function and for preserving the ionic gradient across the cell membrane, membrane potential, and osmotic equilibrium, thus allowing the transportation of Na+, K^+, Ca^{2+} and Mg^{2+} ions across the membrane (Table 3).

The photomicrograph section of the ovary in the DHEA exposed rats shows an increasing number of cystic follicles with a thicker theca cell layer, a marked higher level of collagen especially in the region around the follicle, there is absence of corpora lutea and increased expression of Bax apoptotic protein. The group treated with Vitamin C shows normal histo-architecture of the ovary and the interstitial tissues are also normal. While the group treated with both Vitamin C and DHEA revealed a decrease in a number of cystic follicles with a thinner theca cell layer, a reduced level of collagen especially in the region around the follicle, increased expression of BCl_2 anti-apoptotic protein and E-Cadherin adhesion molecule (Plate A–P).

Limitations: Further studies should be conducted to investigate other contributory pathways and several other regulatory genes causing these disorders. There are limitations in the form of a small sample size and short follow up.

Strength: The biochemical parameters, immunohistochemistry and mRNA androgen receptor gene expression been affected by oxidative pathway shows sufficient evidence and highlights the importance of these pathways; therefore, attention should be given to this area when considering treatment options.

5. Conclusion

Vitamin C ameliorated the effects of PCOS in Wistar rats. It may be explored as a potential agent to overcome the pathophysiology linked with PCOS because it improves ovarian morphology and anovulation associated with PCOS.

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