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Vitamin C reduced *Pausinytalia Yohimbe induced* reproductive toxicity in female Wister rats

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Abstract

Medicinal plants have been used as a source of ailments healing for several years and had gained acceptance as well as widespread use in many cultures of the world. *Pausinytalia Yohimbe (P.yohimbe*) had been used in folklore medicine by tribes in Nigeria as aphrodisiac. Despite its beneficial effects as an aphrodisiac, we had demonstrated that chronic use of *P.yohimbe* had adverse effects on both male and female reproductive systems as evidenced by its effects on hormonal and structural changes in the reproductive systems. This study was done to determine if Vitamin C a potent anti oxidant can ameliorate the toxic effects of *P.yohimbe.*

Twenty female Wistar rats weighing 160 -200g with normal estrus cycles were randomly allotted into four experimental groups of five rats each. Group A was the control group while groups B, C and D served as test groups. Group A received distilled water while group B received 5% Vitamin C only, group C received 150mg/kg body weight and D received vitamin C and 150mg/kg body weight of aqueous extract of *P. yohimbe* respectively for 4 weeks via oral gavage. Estrus cycles of the rats were evaluated daily and were sacrificed after 4 weeks. Blood samples obtained were used for hormonal assay (follicle stimulating hormone (FSH) and Luteinizing hormone (LH), estradiol and progesterone). Ovaries and uteri removed were used for Heamatoxylin and Eosin staining for histology.

Vitamin C restored normal estrus cycle, improved gonadotropins (FSH and LH) production, and also ameliorated the degenerative changes in both ovaries and uteri of rats treated with *P. yohimbe*. Vitamin C had no significant effects on estradiol and progesterone production of rats treated with *P. yohimbe*.

We concluded that Vitamin C ameliorated *P. yohimbe* induced reproductive toxicity in female Wistar rats. It may be that the mechanism of *P. yohimbe* induced reproductive tissue destruction and gonadotropins decrease is via oxidative stress. Vitamin C may be recommended for those chronically exposed to *P. yohimbe* for prevention.

Keywords: *Pausinytalia Yohimbe,* Reproductive hormones, Reproductive toxicity, Vitamin C, Wister rats

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Introduction

Medicinal plants have been used as a source of ailments healing for several years and had gained acceptance as well as widespread use in many cultures of the world (Hoareau and Dasilva 1999, Hassan 2012). Medicine plants have been used to treat different kinds of disease conditions including hypertension, diabetes, infertility etc. Different tribes and cultures all over the world have also used medicine plants as a source of aphrodisiac that arouse sexual desire (Singh et *al.* 2013; Opuwari and Moundipa, 2021) even in women. Several other plants have been associated with arousal of sexual desire too and they include *Pausinytalia Yohimbe_Phoenix dactylifera, Fadogia agrestis, Montanoa tomentosa, Chione venosa and Butea frondosa* (Malviya et *al.* 2011). *P.yohimbe* is a plant that had been used also in folklore medicine by tribes in Nigeria as aphrodisiac (Yakubu et *al.* 2007). Despite its beneficial effects as an aphrodisiac, we had recently demonstrated that chronic use of *P. yohimbe* had adverse effects on the male reproductive system affecting reproductive hormones and structural changes in the male (Ajonuma *et al*. 2017a, Ajonuma *et al* 2017b). We further demonstrated that in female rats, it causes degenerative changes in both uterus and ovaries (Ajonuma *et al.* 2018). The common pathway observed for reproductive dysfunction was via oxidative stress.

Vitamin C is a water-soluble vitamin that plays essential role in physiological protection of organs as a powerful antioxidant (Saygin et al. 2018). Vitamin C as an antioxidant had been used to restore reproductive functions and anomaly in reproductive systems in various species (Saygin et *al*. 2018; Laili et *al*.2015).

This study evaluated the ameliorative effects of vitamin C on P. *yohimbe* induced reproductive toxicity in female Wister rats.

Materials and Methods

Animals

Twenty female Wistar rats weighing 160 -200g, were obtained from the Animal House of Lagos State University College of Medicine (LASUCOM), Ikeja, Lagos State, Nigeria. The rats were fed with standard rat chow obtained from Agege Livestock Feed Mills, Agege, Lagos, Nigeria and water *ad libertum*. The animals were kept in an environment of 12 hours dark and 12 hours light cycle and at room temperature. They were allowed to acclimatize for 2 weeks prior to the study. Ethics approval was obtained from Animal Research Ethics Committee of Lagos State University College of Medicine and its rules and regulations for animal experimentation were strictly adhered to.

Preparation of *P. yohimbe extract* and Vitamin C

Dried berks of *P. yohimbe* were used in this study. They were purchased from an herbal merchant in So Kasua, Gusau, Zamfara State, Nigeria. Other local merchants also confirmed the stem as *P. yohimbe*, identifying the specie as “Dan Cameroon”.

P. yohimbe extraction has been previously described (Ajonuma *et al*. 2016a, Ajonuma *et al* 2016b). Briefly, the bark of *P. yohimbe* were pounded in a wooden mortar and then blended in an electric blender to a fine smooth powder. 150g of powdered *P.yohimbe* was boiled in three liters of distilled water in a beaker on a hot plate with a magnetic stirrer (Hot Plate with Magnetic Stirrer, for 90 minutes at a temperature of 100°C. The boiled extract was allowed to cool, and then filtered through a filter paper and placed in oven at about 100°C for 3 days to dry. The oven dried extract was administered to the rats at a dose of 150mg/kg of body weight.

Vitamin C (KUNIMED Pharmachem Limited, Nigeria) was prepared by dissolving 5g of Vitamin C in 100mls of distilled water.

Experimental Design

The experimental animals were randomly allotted into 4 experimental groups. Group A was the control group while groups B, C and D served as the test groups. Each group consists of 5 rats. Group A received distilled

water and while group B received vitamin C only. Group C received 150mg/kg body weight and D received 5% of vitamin C and 150mg/kg body weight of aqueous extract of P. *yohimbe* via oral gavage for 4 weeks. After 4 weeks of administration, the rats were anaesthetized using ketamine HCL. Blood samples collected via cardiac puncture were stored in plain sample bottles for hormonal assay in -20ºC until used. Uterus and ovaries removed from each rat were weighed, fixed and stored in 10% buffered formalin for Hematoxilin and Eosin (HE) staining.

Serum Preparation

The collected blood samples were placed in a tabletop centrifuge and were centrifuged for 20mins at 3000rpm; the separated supernatant was separated by use of micropipettes and placed in a clean sample bottle which was stored at -20^{0}C until used.

Hormonal Assay

The reproductive hormones; Follicle stimulating hormones (FSH), Luteinizing hormones (LH), Estradiol and Progesterone concentrations were determined quantitatively using enzyme linked immune-sorbent assay (ELISA) kits as outlined in the manufacturer's manual. The serum hormone concentrations were then determined from their respective calibration curves.

The microwells were formatted for each serum reference. 25ul of enzyme reagent was added to the wells. 100ul of enzyme conjugate was added. The micro wells were swirled gently for 20secs to enable mixing. The micro wells were covered to incubate for 60 minutes at room temperature. After incubation, the content of the micro wells was discarded. 350ul of wash buffer was added and washing was repeated for a minimum of five times and blotted on an adsorbent paper. 100ul of substrate solution was added to each well. The microwells were incubated at room temperature for 20 minutes. 50ul of stop solution was added to each well and was gently mixed for 20 seconds. The solution was read within 30minutes, and each well was read at 450nm using a reference wavelength of 630nm on a STAT Fax 4700 ELISA microwell strip reader (Stat Fax Awareness Technologies, USA).

Haematoxylin and eosin (HE) staining

This was carried out as described by Akpantah *et al*. (2003). In this procedure, the collected organs previously stored in 10% formalin were cut in slabs of about 0.5cm thick transversely and fixed in 10% formalin for a day after which it was transferred to 70% alcohol (ethanol) to cause the transversely cut tissues to become dehydrated. The tissues were passed through 90% of alcohol and chloroform for different durations before they were eventually transferred into two changes of molten paraffin wax for 20minutes each in an oven of 57^{0}C. Serial sections were cut using rotary microtome at 5microns. Slides were prepared from these tissues. The slides were then de-waxed and passed through absolute alcohol (2 changes); 70% alcohol and then to water for five minutes. The slides were then stained with haematoxylin and eosin. Photomicrographs of stained slides were captured using a xxx camera attached to a xxx microscope.

Determination of Estrus Cycles of rats

Estrus cycle was determined as earlier described by Ajonuma *et al.* (2018). Briefly, cotton bud swab wetted with normal saline was carefully inserted into the vagina of the restrained rats. The swab was then gently turned and rolled against the vaginal wall and removed. Cells obtained were transferred to a dry glass slide by rolling the swab across the slide. The slides were air dried, and fixed using methanol. It was allowed to dry and then dipped into Field stain A for 15 times and Field stains B for 8 times. The slide was then dipped into distilled water to wash off excess stain and allowed to dry. Each slide was examined under microscope to determine the estrous cycle stages. Vaginal smear of the animals was taken daily for 12 days after the third week of *P. yohimbe* administration.

Statistical Analysis

Data are presented as mean and standard error of mean (SEM). Analysis of variance was use for data assessment and differences between groups were compared using Turkey comparison test. A p-value of ≤ 0.05 was considered significant. Statistical analysis was carried out using Graph Prism Software Version 8.01, Graph Pad Inc. San Diego, CA, USA.

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Results

Effect of vitamin C on Estrus Cycles of rats

Estrous cycle synchronization occurred among rats in group A (control). There were little or no changes and in the progression from estrous to other stages of cycles. However some rats presented longer cycles in group A (control). Estrous cycle synchronization also occurred among rats in group B (vitamin c). As in the control group, some rats presented longer cycles. There were no significant differences in the progression from the estrous cycle stages when compared to group A (control). Estrous cycle synchronization also occurred among rats. However some rats presented longer cycles and irregular cycles, by keeping in the same phase during 4-5days. There is significant different in the progression of the estrous cycle stages when compared to Group A (control). Estrous cycle synchronization occurred among rats. However some rats presented longer cycles and irregular cycles, by keeping in the same phase during 4-5days (Figure 1).

Effect of vitamin C on body weight changes in *P. yohimbe P. yohimbe* treated rats

Vitamin C caused significant weight loss ($p<0.05$) in *P. yohimbe* treated rats when compared with the control. *P. yohimbe* treated rats also exhibited weight loss. It appears *P. yohimbe* and vitamin C had synergistic in inducing weight loss (Figure 1.1).

4.2 Effect of vitamin C on ovary weight of *P. yohimbe* treated rats

Vitamin C caused improvement in weight of the ovaries of *P. yohimbe* treated rats when compared with rats treated with *P. yohimbe* alone although the improvement was not statistically significant ($p>0.05$). The group treated with *P. yohimbe* alone had the lowest ovary weight among the groups (Figure 1.2).

4.3 Effect of vitamin C on uterine weight of *P. yohimbe* treated rats

Vitamin C caused improvement in weight of uterus of *P. yohimbe* treated rats when compared with rats treated with *P. yohimbe* alone, although the improvement was not statistically significant. The group treated with *P. yohimbe* alone had the lowest uterine weight among the groups (Figure 1.3).

4.4 Effect of vitamin C on Reproductive hormones of *P. yohimbe* treated rats

Table 1 showed vitamin c improved the serum levels of both FSH and LH hormones suppressed by *P.* yohimbe in the female rats, although these changes were not statistically significant. Vitamin C seems to further suppress the production of estradiol and indeed increased the secretion of progesterone in *P. yohimbe* treated rats, but these changes were not statistically significant.

4.5 Effect of vitamin C on Histology of ovaries of *P. yohimbe* treated rats

Vitamin C ameliorated the degenerative changes seen in *P. yohimbe* treated rats as evidenced by near normal structure of rats treated with both Vitamin C and P. yohimbe when compared with rats treated with *P. yohimbe* alone. The histological abnormalities seen in *P. yohimbe* treated rats include tissue edema, destruction and lack of oocytes in matured grafian follicles Figure 2.

4.6 Effect of vitamin C on estrus cycle *P.yohimbe* treated rats

The estrus cycle were regular was no significant changes in the progression of the estrous cycle of rats in Groups A (control) and Group B (vitamin C treated). Both the *P. yohimbe* treated group and the group given both *P. yohimbe* and Vitamin C had prolonged irregular estrus cycles.

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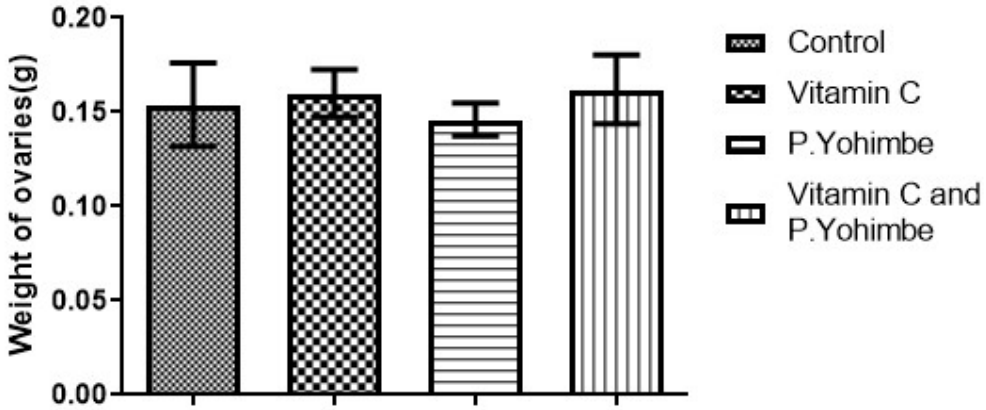

Figure 1.1. Change in body weights of rats following administration of Vitamin C and *P. yohimbe* *p<0.05, n=5 rats per group

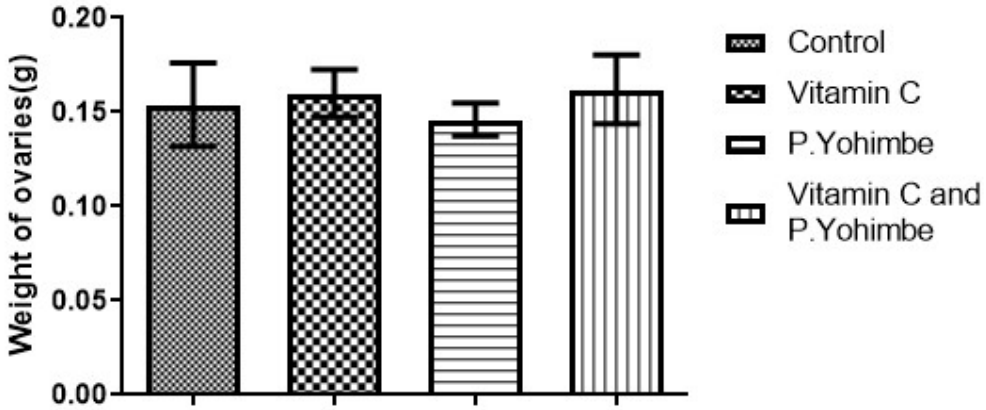

Figure 1.2. Effect of administration of Vitamin C and *P. yohimbe* on the weights of ovaries of rats

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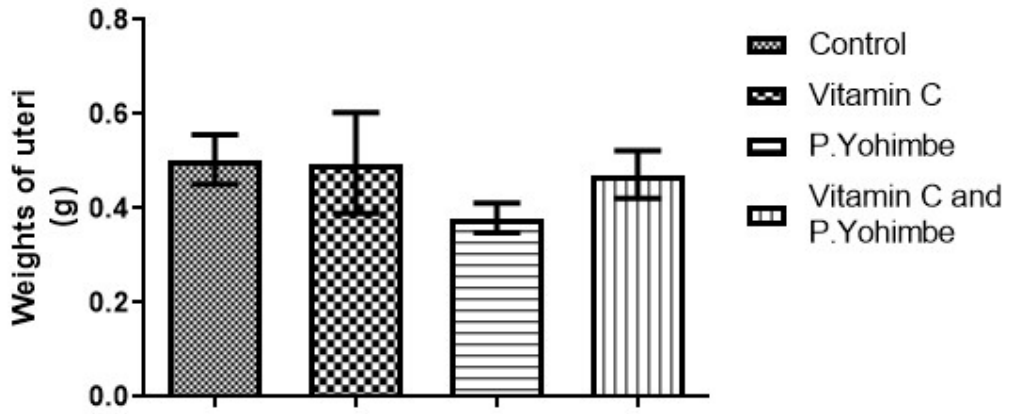

Figure 1.3. Effect of administration of Vitamin C and *P. yohimbe* on the weights of Uteri of rats

Table 1. Effect of administration of Vitamin C and *P. yohimbe* on the Reproductive Hormones

Reproductive Hormones	Group A (Control)	Group B (Vitamin C)	Group C (*P.yohimbe*)	Group D (*P.yohimbe* and Vitamin C) *P. yohimbe*
Follicle stimulating hormone (FSH) (µ/ml)	0.28±0.09	0.2±0.04	0	0.38±0.38
Luteinizing hormone (LH)(µ/ml)	1.46±1.06	0.59±0.54	0.04±0.02	0.18±0.12
Estradiol (pg/ml)	225.3±16.11	217.4±19.7	192.8±5.81	186.9±5.07
Progesterone (ng/ml)	34.13±8.78	59±6.64	36.4±8.37	47.75±9.62

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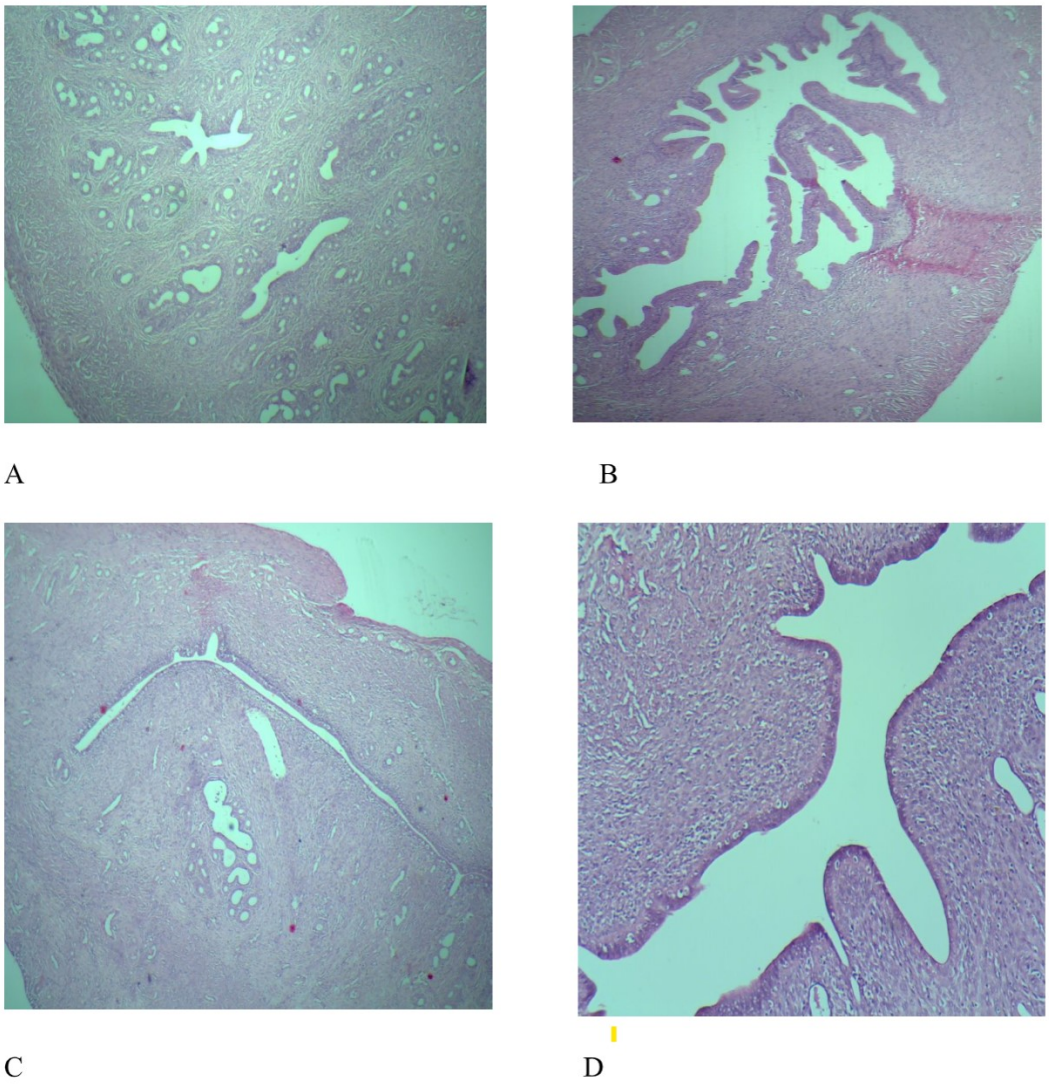

Firgure 2 : Effect of P yohimbe on the Uterus

(A= Uterus of control group), (B= Uterus of test group B (vitamin C), (C=Uterus of test group C (*P.yohimbe*) and (D=Uterus of test group (*P.yohimbe*+vitamin C). (Magnification X10).

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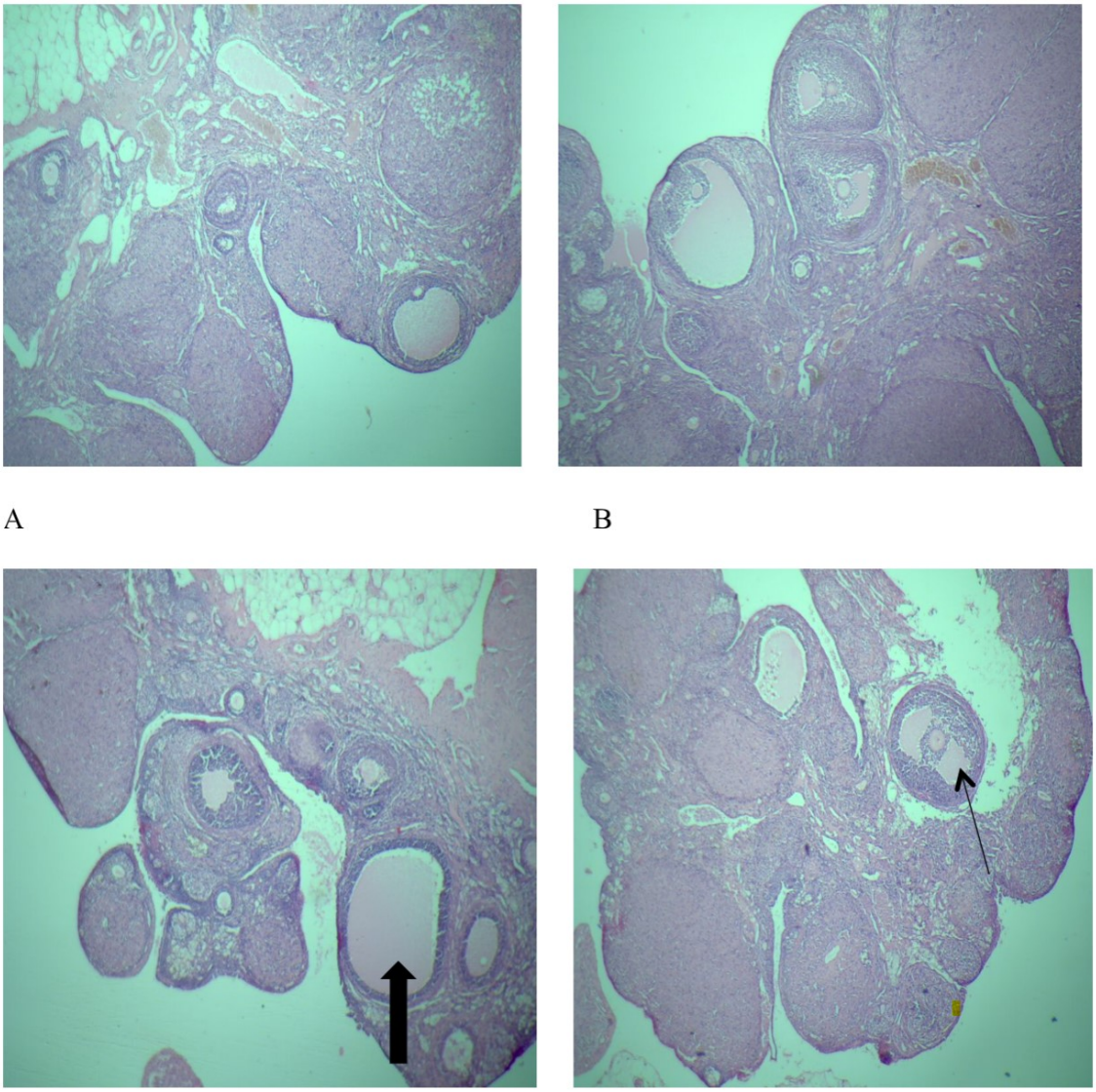

Figure 3: Effect of of P.yohimbe on ovaries

(A= ovary of control group; where arrows indicated secondary follicle and grafian follicle with ovum), (B=ovary of test group; where arrows indicated secondary follicle and grafian follicle with ovum), (C= ovary of test group; where an arrow indicated degeneration of follicles, thick arrow indicated lack of oocytes in the matured graafian follicle), (D=ovary of test groups; where thin arrow indicated recovery of ovum) (Magnification X10).

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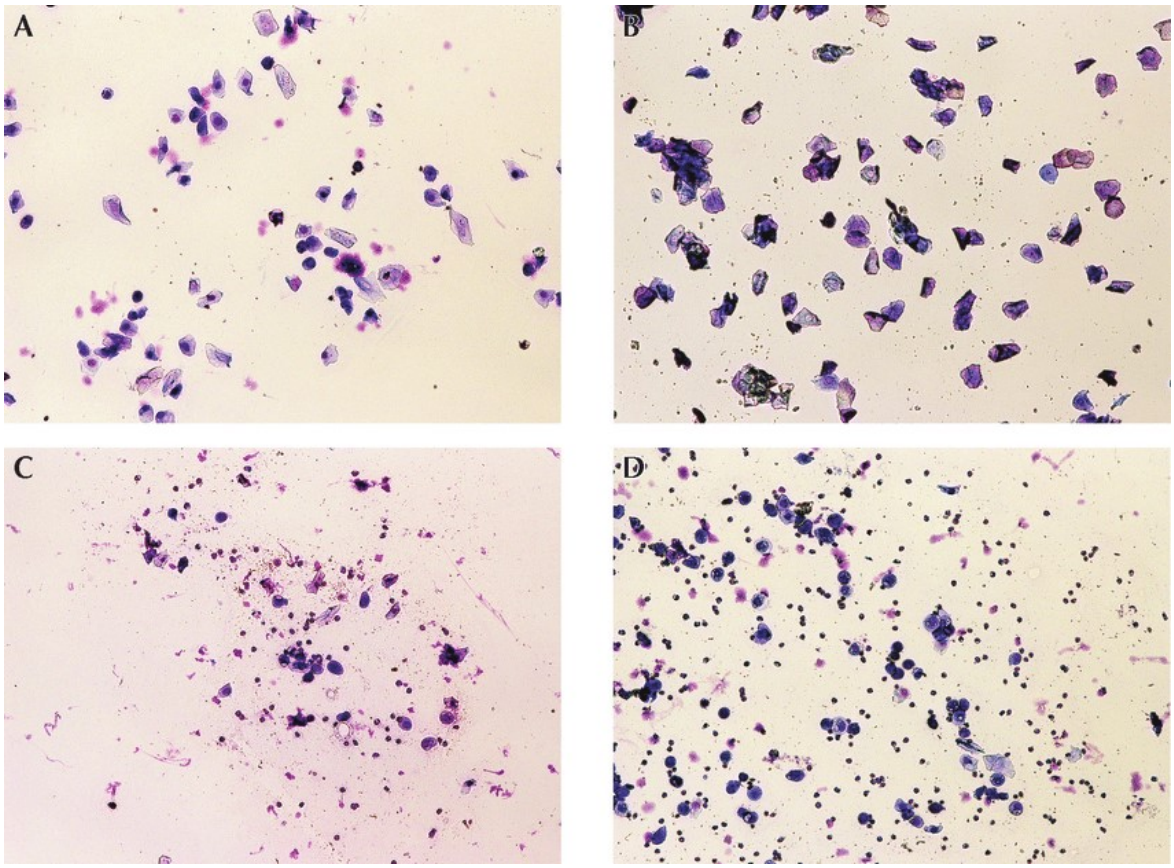

Figure 4.

Photomicrographs of vaginal cytology of Wistar rats in different Estrus stage cycles. *P.yohimbe* did not induce difference changes in the morphology of the phases despite phase delays. A: Proestrus B: Estrous C: Metestrus D: Diestrus (Magnification X10). Proestrus consists of predominantly nucleated epithelial cells. Estrous primarily consists of anucleated cornified cells. Metestrus consists of same proportion of leukocytes, cornified and nucleated epithelial cells. Diestrus mainly consists of leukocytes.

Discussion

This study evaluated the effect of vitamin C on *P. yohimbe* induced reproductive toxicity in female Wistar rats. P. *yohimbe* caused a significant weight loss when compared with control. The finding of weight loss in these rats is in line with previous studies (Ajonuma *et al*. 2017a, 2017b and 2018) and the effect of vitamin C in causing weight (Johnston 2005: Johnston *et al.* 2013: Garcia-Diaz *et al.* 2014). Vitamin C may mediate weight loss by modulating adipocyte lipolysis, regulate glucocorticoid release from adrenal glands, inhibit glucose metabolism and leptin secretion on isolated adipocytes, leading to an improvement in hyperglycemia and decrease glycosylation in obese-diabetic models; and reduce the inflammatory response and oxidation of fats (Johnston 2005, Johnston *et al.* 2013, Garcia-Diaz *et al.* 2014). Weight loss however has been associated with improvement in reproductive outcome among females (Dag and Dilbaz, 2013).

The study demonstrated that Vitamin C supplementation ameliorated the uterine weight loss as well as degenerative changes induced by *P.yohimbe* in the uterus. Normal uterine epithelial structure is important for implantation of fertilized oocytes and therefore may play a vital role in fertility. This finding agrees with earlier work that vitamin C protect the uterus via protecting endometrial toxicity (Laili *et al.* 2015).

Vitamin C administered to *P.yohimbe* treated rats also caused improved weight of the ovaries as well as amelioration of degenerative changes seen in the ovaries documented in previous studies in our laboratory. The *P.yohimbe* treated rats that had some follicles without oocytes (empty follicles) were restored in rats treated with

Vitamin. These findings of the ability of vitamin C to restore structural defects have earlier been reported by Olaniyan *et al.* 2019.

Vitamin C improved the secretion of gonadotropins in *P. yohimbe* treated rats. *P. yohimbe* caused suppression of gonadotropins (follicle stimulating hormone and luteinizing hormone) in female rats as seen in previous studies ( ) but was greatly ameliorated by Vitamin C this study. However, these improvements were not significant in all groups treated with Vitamin C and *P. yohimbe*. Interestingly, Vitamin C has been reported to improve the secretion of gonadotropins in several conditions (Okon and Utuk, 2016). Improved gonadotropins secretion is very important as these pituitary hormones drive folliculogenesis and ovulations in females.

Vitamin C seem to reduce estradiol levels in *P.yohimbe* treated rats when compared with the controls. The implication of this finding is not known but these findings suggest further research is needed. Vitamin C caused improved progesterone production in *P.yohimbe* treated rats, although this finding was not statically significantly. The effect of vitamin C on the progesterone levels may play a role in reproductive toxicity treatment as it will increase the receptivity and preparation of the endometrium to the fertilized oocyte (Aboud 2014). The elevated progesterone may account for the improved uterine seen in female rats treated with both Vitamin C and *P.yohimbe*. The restoration of normal estrus cycle by vitamin C in *P.yohimbe* treated rats may also have been mediated by the ability of vitamin C to improve ovarian structure and improve the secretion of some hormones.

The effect of Vitamin C in restoring reproductive function is similar to the effect of antioxidant like thymoquinone in ameliorating the effect of reprotoxic agent (Yaghutian et al 2021). Future studies should be directed towards evaluating the effect of other antioxidants in mitigating the effects of P.yohimbe.

In summary, the restoration of normal estrus cycle, improvement of degenerative changes in ovaries and uteri, improved gonadotropins (FSH and LH) production and improved reproductive functions in *P.yohimbe* treated rats by Vitamin C probably suggested that *P.yohimbe* induced reproductive abnormalities are via oxidative stress. Anti oxidants supplementation in those exposed to *P.yohimbe* may be helpful as a preventive measure.

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