Original Article

Effect of Propolis Supplementation on Structural Histomorphology of the Pituitary-ovarian-uterine Axis in Rats

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ABSTRACT

Background: Propolis (PRO) is a natural animal product used in multiple applications such as wound healing, pharmaceutical products, and food production. It has high nutritional and medicinal content, and numerous biological properties, such as antioxidants (AOs) and anti-inflammatory effects.

Objectives: The purpose of this study was to assess the impact of PRO supplementation using histomorphometric and morphological examinations on the rat liver, pituitary glands, ovaries, and uterus.

Methods: Four groups (G) of albino rats were randomly assigned, with each group consisting of six rats. The groups G1, G2, G3, and G4 were administered PRO at progressively higher doses of 0, 150, 300, and 500 mg/kg body weight daily, respectively. The period of treatment was 28 days. Blood specimens were taken from the anesthetized rats' hearts that were sacrificed during the proestrus stage. For morphological and histological analyses, the uterus, ovaries, pituitary, kidney, and liver were removed.

Results: The data demonstrated significant ovarian structural and histological changes in the PRO-treated groups, including an increase in both interstitial cells and growing follicles, as well as a significant rise in the histological structures of pars distalis. The findings indicated that the G3 and G4 groups exhibited an increase in the height of the uterine luminal and glandular epithelia, as well as proliferation of the uterine glands, in contrast to the G1 and G2 groups. This study revealed that hepatorenal histomorphology is normal and that the number of Kupffer cells decreased in the treatment groups. Moreover, the serum of the G4 group had a substantially greater concentration of estradiol (E2) than that of the G1 group. In this study, non-pregnant rats' levels of oxidative stress were decreased and their AO and total AC capabilities were raised by PRO.

Conclusion: PRO induces the proliferation of the endocrine cells in the pituitary gland, as well as in ovarian and uterine tissues, as evidenced by the elevated E2 concentrations and improvement in enzymatic TAC defense in the cells.

Keywords: Antioxidant (AC), Ovary, Propolis (PRO), Pituitary, Uterus

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Introduction

Propolis (PRO) is a dark-colored substance, which is secreted from the saliva of bees and is commonly well-known as bee glue, which is composed of exudate collected from various plant parts, including flower buds, leaves, and stems, as well as secretions from the salivary glands. PRO, which derives from the Greek word that means defense community, is an ancient name (Kocot et al., 2018; Touzani et al., 2019). The use of PRO for various purposes stems from its biological characteristics, which include antioxidative, anti-inflammatory, anti-carcinogenic, anti-allergic, immunostimulant, and tissue regeneration properties. Its long history dates back to at least 300 BC, and it has been used as medicine ever since, even to the present day (Nakamura et al., 2010; Salrian et al.,2022; Toutiaee et al., 2023). It has long been believed to be a potent remedy that the oriental community has utilized for generations to treat a variety of illnesses. Recent years have seen increased interest in research on several aspects of PRO, including its nutritional and health benefits. Scientific explanations largely support these traditional beliefs. Previous reports have confirmed that PRO protects rats against the hepatorenal and reproductive damage caused by metals (Sajjad et al., 2020; Okail et al.,2020). Caffeic acid, phenethyl ester, artepillin C, and chrysin, which are components of PRO, are responsible for their biological activity against cancer in cell lines related to the gastrointestinal tract, the respiratory system, and the female reproductive system (Yildirim et al., 2016; Dadar et al., 2022).

Improving libido and other behavioral aspects of reproduction are among the conventionally accepted advantages of PRO that need to be validated by scientific research. PRO is a complex phytocompound, created when bees collect balsamic and resinous materials. It has a high concentration of polyphenols, amino acids, polysaccharides, steroids, terpenoids and phenylpropanoids, aldehydes and ketones, and many other organic and inorganic compounds (Zulhendri et al., 2021). PRO has been identified in earlier research to have regenerating and regulating properties (Meghalatha et al., 2024). These biological properties of PRO can potentially influence reproduction and fertility. Studies conducted on the effects of PRO in areas other than reproduction explain the important biological characteristics of PRO, which may indicate its impact on fertility and reproduction. For example, Zulhendri et al found that reproductive hormones, such as steroid hormones, were present in PRO after studying its composition. The researchers concluded that PRO can be used as estrogen treatment to alleviate aging caused by ovariectomies (Zingue et al., 2017). This research supported earlier studies' conclusions that PRO includes steroid hormones. In their investigation of the protective effects of PRO supplementation against osteoporosis in ovariectomized rats, Kaya et al. (2022) also noted that the serum estradiol (E2) concentrations of these rats were comparable to control rats. This finding may help postmenopausal women at high risk of osteoporosis and demonstrates the compensatory role of PRO extract for E2 in the absence of the ovary. Other biological characteristics of PRO might have an impact on mammalian fertility and reproduction. Research on the application of PRO in the management of uterine fibroids has revealed that this treatment is novel (Ali et al., 2018). Furthermore, PRO has been demonstrated to be a useful treatment for improving histopathological results in polycystic ovary syndrome (Sapmaz et al., 2022).

A previous study showed that oral administration of PRO to ovariectomized rats dramatically increased the weight of the uterus and uterine structures, such as the thickness of the luminal epithelium (LE), compared to the corresponding values in the control group (Okamoto et al., 2015). PRO improves the generation of steroid hormones like E2 and serves as an antioxidant (AO), which are molecules that can shield cells from the damage caused by free radicals, according to recent research findings on PRO in fields other than reproduction (Kaya et al., 2022). PRO includes AOs, like flavonoids, phenolic acids, and terpenoids (Kocot et al., 2018).

Trirtepernoids, which are used to treat gynecological problems and are known to have estrogenic properties, as well as derivatives of caffeic acid, have been found in PRO. Additionally, PRO possesses estrogen-like properties in vivo and may treat hot flashes and vaginal dryness, which are menopausal problems (Zingue et al., 2017).

The purpose of this study was to assess the impact of PRO supplementation on female reproduction. Rats were given varying amounts of PRO extracts, and their reproductive organs were analyzed both grossly and histomorphometrically. Additionally, reproductive parameters and related biomarkers, like hormone (E2) levels, were identified using a standard technique. We also evaluated oxidative status, including oxidative stress and AO levels.

Materials and methods

Preparation of PRO

PRO was purchased from the market, and the PRO extract was prepared according to the method by Hendi et al. (2011). Therefore, 10 g of PRO was soaked in 100 ml of distilled water in dark brown container and left for 24 hours at 25 ˚C. The container was shacked every 2 or 3 hours daily for two weeks using a hot plate (45 ˚C) with a magnetic stirrer and was kept in a dark place. Ultimately, the PRO solution was allowed to cool to 25 ˚C before the rats received doses determined by their body weights.

Methods

Animals

Twenty-four adult female albino rats (12 weeks old, 160-210 g) were obtained from the Animal Resources Centre. Following a 14-day acclimatization period under standard laboratory conditions (controlled temperature and humidity), the rats were provided with ad libitum access to a standard rodent diet and water. All animal procedures were conducted in accordance with the ethical guidelines of the Institutional Animal Care and Use Committee at the University of Kufa.

Experimental design

To minimize bias and the influence of confounding factors, the rats were randomly assigned to four treatment groups (G1-G4, n=6/group) using a randomized allocation method. This ensured that each rat had an equal probability of being assigned to a particular treatment group.

Treatment groups:

G1: Control group (0 mg/kg PRO)

G2: Treated group (150 mg/kg PRO)

G3: Treated group (300 mg/kg PRO)

G4: Treated group (500 mg/kg PRO)

PRO was administered orally via gavage daily for four weeks. The dosage levels were selected based on previous studies (Teles et al., 2015; Salehi et al., 2022; Sheir et al., 2023).

Euthanasia: At the end of the four-week treatment period, rats were euthanized using CO_2 asphyxiation. Prior to euthanasia, animals were anesthetized with a combination of ketamine (30 mg/kg) and xylazine (10 mg/kg) during the proestrus stage.

Vaginal cytology

At the start of the trial, the rats were synchronized and given two intraperitoneal doses of 0.5 mg of estrumate, separated by three days (Pallares, 2009). Every day for the duration of the trial, vaginal smears were collected from each animal for four weeks to assess the regularity of their estrous cycles, according to Albishtue et al. (2018a). Light microscopy was used for cytological examination.

Histomorphological examinations

The anesthetized rats were sacrificed, and their reproductive organs, including the uterus and ovaries, as well as their kidney, liver, and pituitary glands were removed, weighed, and measured. During a gross examination, any noticeable abnormalities were recorded. Fixation of the histology samples was done by 10% formalin for 24 hours. Ethanol dehydration was used for processing fixed tissue samples. Next, sample tissues were immersed in paraffin blocks to bind the sample in a block of wax for section cutting and storage. Ribbon-like sections of the sample tissues were produced by cutting them into 4 µm sections with a microtome. Then, these sections were floated out on a water bath and after that placed on slides of a microscope. These samples were stained with routine stains (Hematoxylin and eosin [H and E]) to evaluate histological structures. Morphometric analysis of the ovaries was carried out with some adjustments in accordance with Albishtue et al. (2018b). Section cuttings were made from the central region to the periphery. Measurements of the thickness of the SOE, the number of ovarian follicles in the whole ovary for each stage, and interstitial cells in a given area were performed using an analyzer images.

Primordial, primary, secondary, and antral follicles were identified by their antral space, cell shape, and number of surrounding layers. Atretic follicles were characterized by fragmentation of the oocyte nucleus, disorganized granulosa cells, and degenerating oocytes (Albishtue et al., 2018b).

The number of uterine glands and histological changes in the endothelium lining of the uteri samples were examined under a microscope (Albishtue et al., 2018a). Uterine samples were used to determine the number of uterine glands and the thicknesses of the luminal and glandular epithelia using the Medical Analyzer Image

(Olympus). The number of pars distalis blood vessels and endocrine cells were counted in an area using the analyzer Image. The number of blood arteries was counted using a 40x objective, whereas the number of endocrine cells was counted using a 100x objective (Albishtue et al., 2018b). Three random measurements per section were made from each animal in all groups. Moreover, the number of Kupffer cells in a given area of the liver was counted using the Medical Analyzer Image with a 100x objective. Moreover, the numbers of Kupffer cells in an area of the liver were counted using the Medical Analyzer Image with a 100x objective. Three microscopic regions of a similar size in each section were examined for statistical analysis (Albishtue et al., 2018a; Albishtue et al., 2020).

Hormone assays

Hormone assays of E2

Animal model and treatment: Female rats were subjected to a 4-week treatment protocol (G1, G2, G3, and G4 were considered treated groups that received graded concentrations of PRO at 0,150, 300, and 500 mg/kg body weight per day, respectively.). The estrus cycle stage was determined daily by vaginal cytology according to the method described by Albishtue et al. (Albishtue et al., 2018a). Animals were sacrificed during the proestrus phase.

Blood collection and serum preparation: 1 mL of blood was obtained from the heart of each rat and immediately transferred to EDTA-containing tubes. The blood samples were then centrifuged at 2500 rpm for 10 minutes at 4 ˚C to collect the serum.

E2 Measurement: E2 levels in serum samples were quantified using a competitive immunoluminometric assay (Manufacturer's kit: Maglumi E2, Maglumi X3, China). Briefly, the assay involves labeling an anti-E2 monoclonal antibody with ABEI and purifying the E2 antigen with FITC. Next, the sample was incubated with the ABEI label, displacing reagent, and calibrators/controls for 10 minutes at 37 ˚C. This was followed by subsequent incubations with the FITC label and anti-FITC-coated nanomagnetic microbeads at 37 ˚C to form antibody-antigen complexes. Then, magnetic separation of the complexes is performed, and the supernatant is removed. Afterward, a flash chemiluminescent reaction is initiated. The relative light units (RLU) is then measured by a photomultiplier, which is directly proportional to the E2 concentration. Finally, E2 levels were determined using a Mindray system.

Effects of PRO on oxidative status

According to Yew et al. (2014) and Schmidt et al. (2014), serum samples were collected and used to analyze oxidative stress biomarkers (OSBs) such as malondialdehyde (MDA), as well as AOs, such as total AO capacity [TAC] and superoxide dismutase [SOD] activity. The lipid peroxidation (LPO) process is generated by polyunsaturated fatty acid peroxidation, resulting in the formation of MDA, which is an indicator of oxidative stress and a physiological metabolite. There is a direct relationship between an increase in free radicals and overproduction of MDA. MDA, a member of the β-dicarbonyl class, is a highly reactive liquid that exists primarily as the enol (Wencel-Delord et al., 2011). The MDA assay kit (Solarbio, China) was used to evaluate the level of LPO in the serum. The EnzychromTM SOD assay kit (Solarbio, China) was utilized to evaluate SOD activity in the serum. This kit measures the percentage of superoxide radicals that undergo dismutation in a particular sample (Schmidt et al., 2014). The TAC was determined using a commercially available kit (Solarbio, China) that measures the number of non-enzymatic AOs present in the sample that inhibit metmyoglobin's oxidation of 2, 29-azino-di-3-ethybenzthiazoline sulfonate.

Statistical analysis

All data were analyzed and presented as Mean±SEM utilizing the Graph Pad Prism software, version 6.0 (San Diego, California). The ratio of uterus and ovarian weights to the body weight and their lengths, the number of uterine glands, interstitial cells, and ovarian follicles in each stage, the thicknesses of ovarian epithelium surface (OES), endothelium, epithelia of the uterine lumen, and uterine glands, as well as histomorphometric parameters in the pars distalis, hormone concentrations, the levels of OSBs and AOs, were compared using an analysis of variance (ANOVA) with Tukey's multiple comparison posthoc test, while the body weights were compared using two-way ANOVA with Bonferroni's multiple comparison tests.

Results

Effects of PRO on vaginal cytology

In the vaginal smears, the three primary cell types observed were leukocytes, nucleated epithelial cells, and cornified squamous epithelial cells. Based on the relative ratios of these cells, the estrous cycle stage of the rat on the day the sample was taken could be identified.

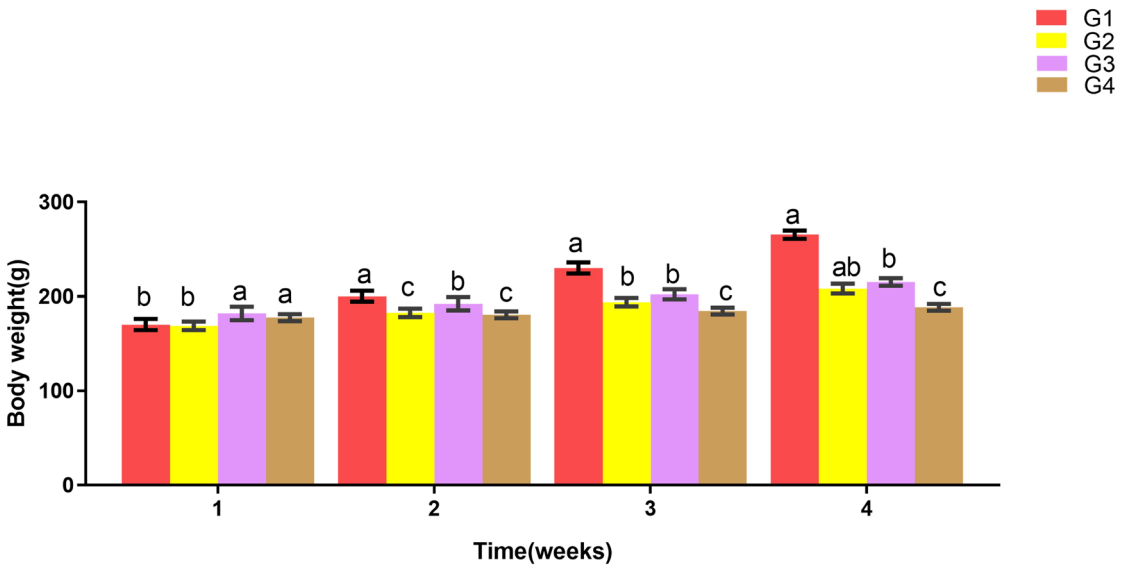

Figure 1. Impact of PRO supplementation on the body weight of non-pregnant rats

Note: PRO supplementation increases body weight loss in rats fed a normal diet. Means±SEM are used to express data. A significant difference at P<0.05 is indicated by different letters a, b, c, and d within rows. The groups that received PRO treatment were denoted by G1, G2, G3, and G4, with their corresponding graded concentrations of 0,150, 300, and 500 mg/kg body weight per day.

The oestrus cycle in rats consists of four stages: Proestrus, estrus, metestrus, and diestrus. At the proestrus stage, we observed rounded, nucleated epithelial cells with oval nuclei and distinctly pale-colored cytoplasms. The rats' estrous cycle lasted four days, as usual.

There were considerable variations in the average body weights of the rats in each group during the study. PRO had a potent weight-reducing impact, depending on the dosage used (Figure 1).

At four weeks of the experiment, ovarian length (P<0.05) and the ovary-to-body weight ratio were larger in the G4 group than in the other groups, while the G1 control group showed lower values (Figure 2A and 2B). In comparison to the control group, the treatment groups exhibited significantly greater uterine lengths and the ratio of uterus weight to body weight, with a peak observed in the G4 group (P<0.05). PRO increased the weights and lengths of the uterus and ovaries in a dose-dependent manner (Figure 2C and 2D).

Impact of PRO on ovarian histomorphology and development of follicles

Rat ovarian representative samples resembled grape-like structures in their gross shape, while Figure 3 shows a histological section of ovarian features. The ovarian surface epithelium (OSE) in each group was of a simple cuboidal type. The cytoplasm of the cells was light and eosinophilic, and their central nuclei were spherical (Figure 4).

The mean number of follicles at each follicular stage and corpora lutea for each treatment group were counted based on the histological study of the rat ovaries, as shown in Figure 5. The mean number of various kinds of surviving ovarian follicles increased in parallel with an increase in the dose of PRO supplementation in all treatment groups. Therefore, in comparison to the control group, the G4 group had significantly (P<0.05) higher mean number of surviving follicles and corpora lutea. Nonetheless, there was no difference in the average number of atretic follicles among all the experimental groups.

Table 1 summarizes the ovarian histomorphometric data for each group. Prolonged use of PRO was associated with dose-dependent increases in both the thickness of OSE and the number of interstitial cells. The highest value for PRO (G4 group) was at 500 mg/kg of body weight. Compared to the control group, the G3 and G4 groups showed a significant increase in interstitial cell count and thickness of OSE. When comparing the treatment groups, it was found that the G2 group had the lowest measurements for both parameters, with the exception of the G3 for the number of interstitial cells, which was significant (P<0.05).

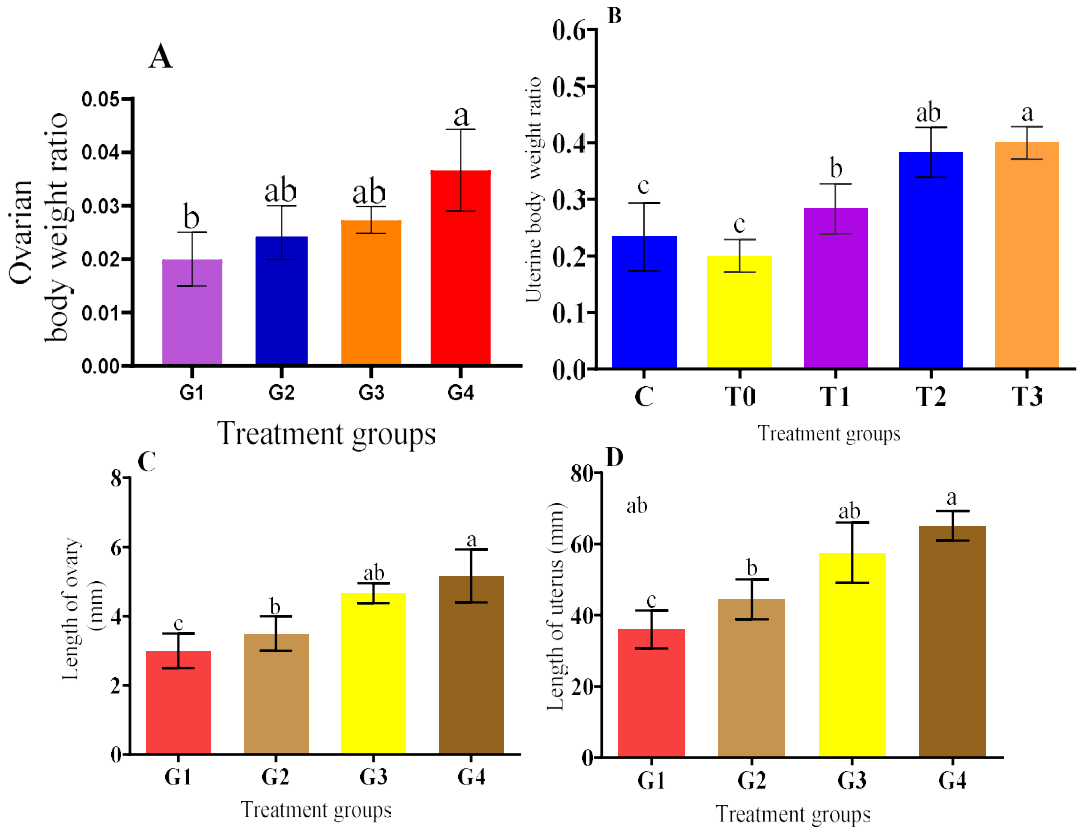

Figure 2. The impact of PRO on the ratio of ovarian and uterine to body weight and alterations in their length in non-pregnant rats

Note: PRO supplementation enhances the weight and length of the ovaries and uterus. Means±SEM are used to express the data. The groups that received PRO treatment are denoted by G1, G2, G3, and G4, with their corresponding graded concentrations of 0, 150, 300, and 500 mg/kg body weight per day.

Effects of PRO on the histomorphology of the pituitary gland

An anatomical study revealed that pituitary glands were disc-like structures in the control and PRO-fed rats (Figure 6). The histomorphology (description of the shape and structure of human and animal tissues) of the pars distalis of the pituitary glands from each group is displayed in Figure 7. The microscopic analysis of the pituitary glands revealed significant development and a higher quantity of active endocrine cells with a higher degree of vascularization in the G4 group compared to the control and other treatment samples, which is similar to the effects seen on follicular development.

Table 2 summarizes the histomorphometric findings for the pituitary glands in each group. Both the number of blood vessels and endocrine cells were found to increase in a dose-dependent manner with PRO. Table 2 demonstrates that in all treatment groups, the quantity of endocrine cells was significantly ($P<0.05$) larger than in the control group. The G4 group had a considerably higher ($P<0.05$) number of cells than the G2 group, while the G3 group had comparable results to the G3 and G4 groups. In addition, the number of blood vessels increased dose-dependently until a substantial increase ($P<0.05$) was seen in the G4 group compared to the control group. The results for the G2 and G3 groups were marginally lower than those for the G4 group but higher than those for the G1 control group.

Effects of PRO on uterine histomorphology

In the proestrus stage, the rat uteri were evaluated. Rat uteri exhibited a duplex morphology. The uterine horns of the PRO-fed and control rats were both normal and regular (Figure 8). Figure 9 and Table 3 present an overview of the uterine histomorphometric data for each group. Dose-dependently, PRO increased the thicknesses of the endometrium, glandular epithelium (GE),

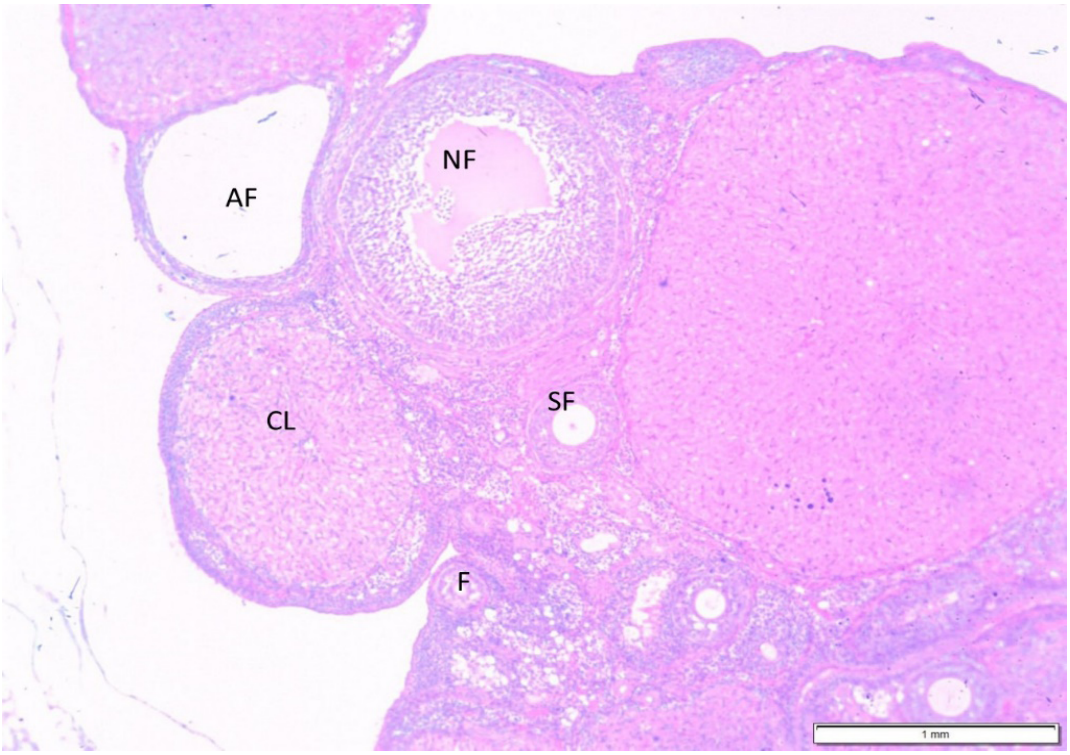

Figure 3. Rat ovarian histological structures showing follicles at various stages, the interstitial cells, and the outer surface epithelium lining the ovary (H&E stain, ×10 magnification)

Abbreviations: F: Follicular unit; SF: Secondary follicle; NF: Antral follicle; AF: Atretic follicle; CL: Corpus luteum.

and LE. The thicknesses of the endothelium, LE, and GE were greater in all treatment groups compared to the control group (P<0.05) (Table 3). The thicknesses of the LE, GE, and endothelium were significantly (P<0.05) greater in all treated groups compared to the controls (Table 3). While the number of uterine glands in the G3 group was similar to those of the G2 and G4 groups, a greater number was seen in the G4 group (P<0.05), which was significantly higher than that in the G1 group. The uterine glands and GE thickness showed a significant dose-dependent increase (P<0.05) when comparing the G4 group to the control group. Results for groups G2 and G3 were marginally lower than those for the G4 group, which were higher than those for the control group (G1).

Effects of PRO on hepato-renal histomorphology

Sections of hepato-renal histomorphology from the four trial groups are displayed in Figures 10 and 11. According to gross and histological examinations, the kidney and liver did not exhibit any abnormal lesions in any area. In contrast to the control group, the treated groups showed a reduced number of Kupffer cells in their livers, as indicated by the histomorphometric data. While the numbers for G3 and G4 were comparable, the lowest number of Kupffer cells was found in the G4 group (P<0.05), which was significantly lower than in the G1 group (Table 4).

Effects of PRO on E2 concentrations

Figure 12 shows how the serum levels of E2 respond to PRO treatment. The concentrations of E2 increased significantly in a dose-dependent manner, with the G4 group recording the highest levels (P<0.05) and the G1 group recording the lowest (P<0.05). Figure 9C demonstrates no discernible variation in the increase in E2 concentrations between the G1 and G2 groups.

Effects of PRO on oxidative status

Figure 13 summarizes TAC, SOD activity, and the MDA levels in the serum of each group. The treatment groups exhibited dose-dependent increases in SOD activity and decreases in MDA levels. By raising the TAC and enhancing the enzymatic AO defense (SOD), these effects altered the redox status. The current study suggests that PRO improves the enzymatic AO defense in the cells.

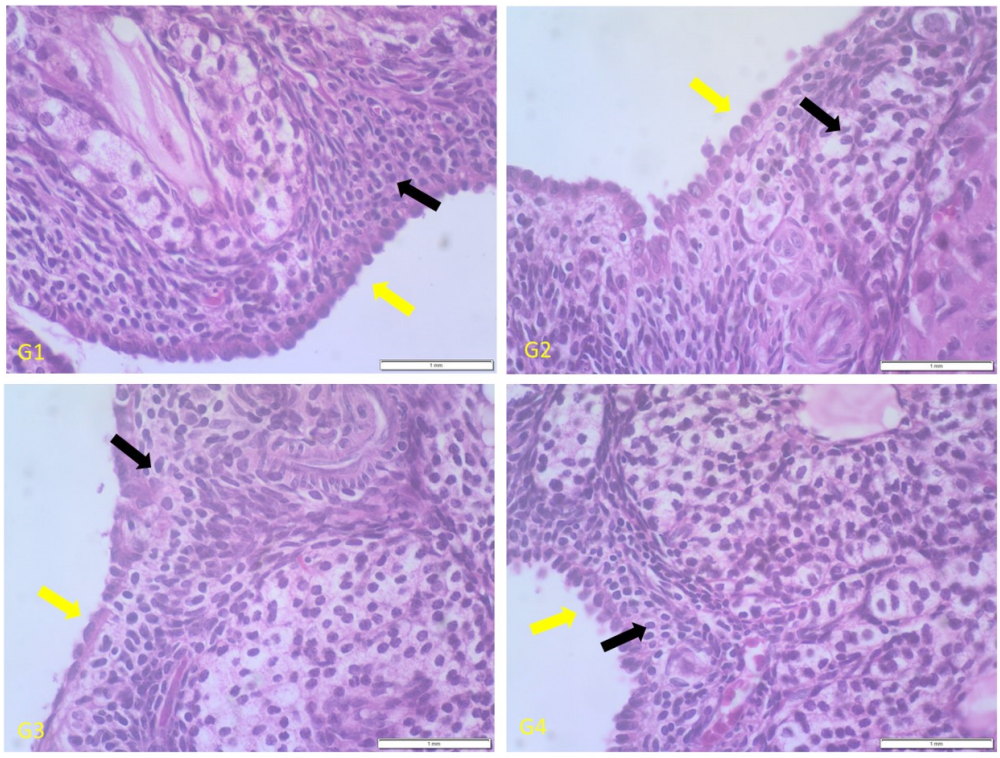

Figure 4. Histological sections of rat ovaries showing the interstitial cells (black arrow) and simple cuboidal epithelium (yellow arrow) in all groups (H&E stain, ×40 magnification)

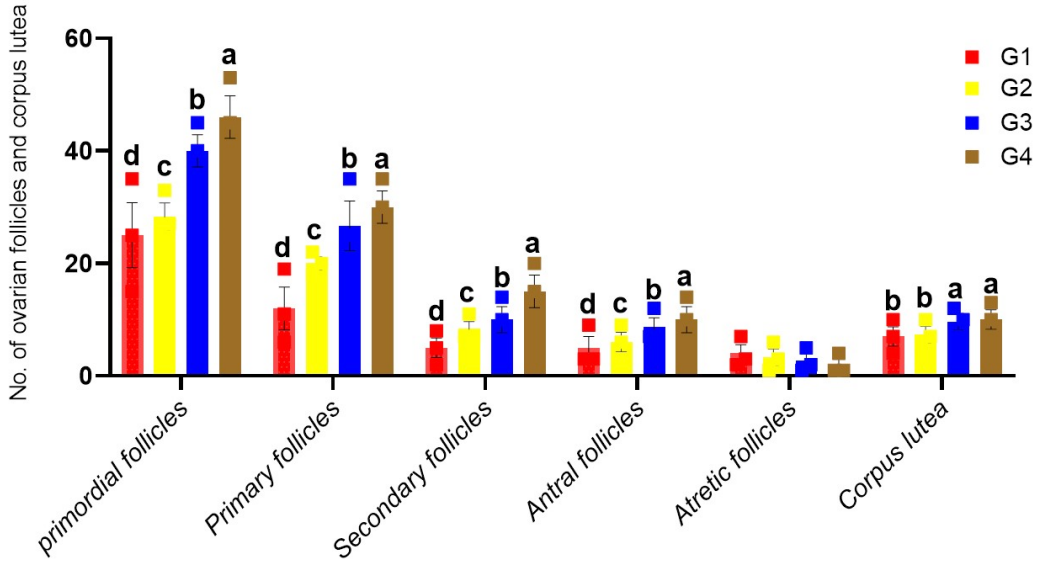

Figure 5. Comparative evaluation of corpora lutea and ovarian follicular development in several groups of rats given varying PRO dosages and sacrificed at the pro-estrus stage

Note: G4 had a significantly (P<0.05) higher number of corpora lutea and a mean number of surviving follicles at all ovarian stages. For every type of follicle and corpus lutea, error bars with distinct letters (a, b, c, and d) denote statistically significant differences (P<0.05).

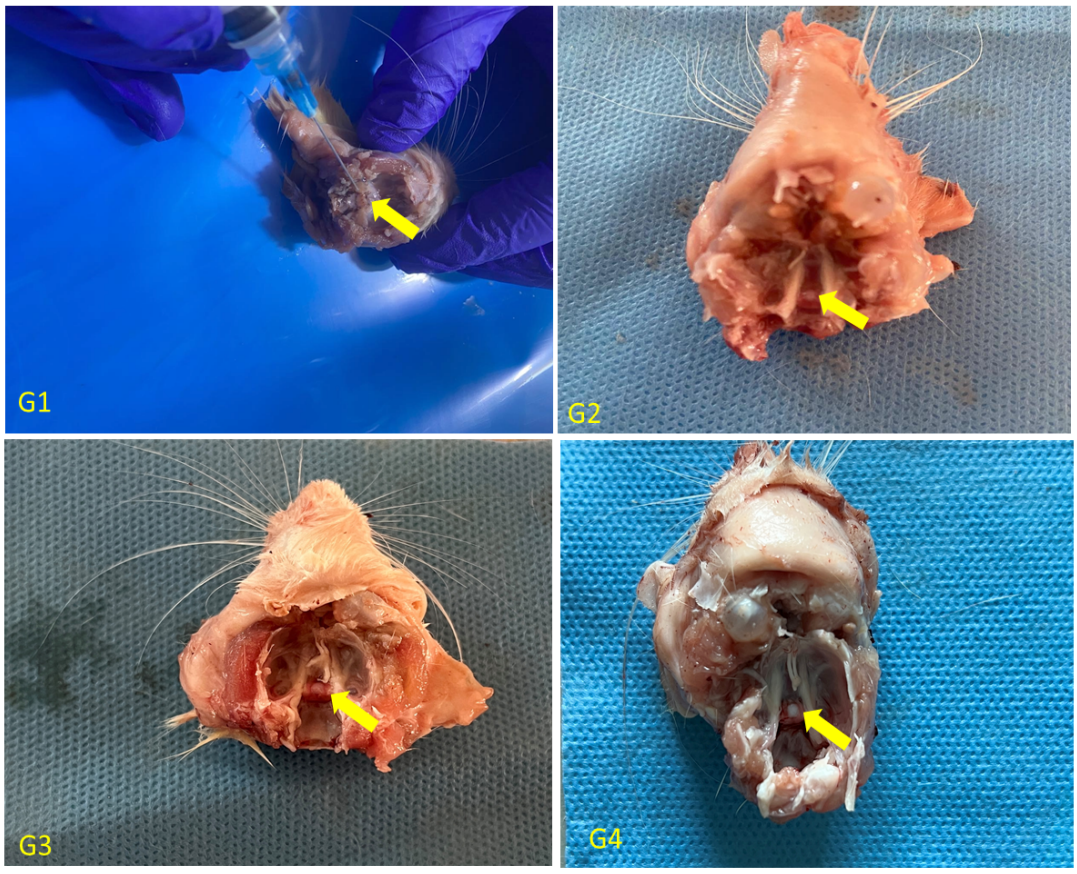

Figure 6. Pituitary glands (yellow arrow) are disc-like structures in their gross shape

Note: All groups showed normal architecture.

Discussion

PRO is a natural animal substance used as hormonal replacement prophylaxis and has regenerating and regulating properties. There is a relationship between endocrine events and alterations in the cell typology of vaginal mucosa. A recent study found that the hypothalamus secretes the gonadotropin-releasing hormone, the pars distalis of the pituitary gland secretes gonadotropins, and the gonads secrete sex hormones, which regulate the normal progression of the estrous cycle (Albishtue., 2018a).

In a previous study, postmenopausal women experienced a markedly increased risk of visceral obesity as a result of altered lipid and carbohydrate metabolism linked to decreased menopause-induced levels of estrogen and progesterone hormones (Donato et al., 2006; Kozakowski et al., 2017). The current findings indicate that the body weights of rats decreased due to PRO dietary components. PRO promotes the inhibition of fat absorption and increases fecal weight, which are mechanisms of weight loss (Sakai et al., 2017). However, other study reveals that PRO is considered a useful natural growth enhancer (Sierra-Galicia et al., 2023).

The current study demonstrated the impact of PRO supplements on increasing the number of surviving ovarian follicles and promoting folliculogenesis in a dose-dependent manner from 150 to 500 mg/kg body weight.

The purpose of the current study was to uncover PRO's potential role in reproduction and its mechanism of action by supplementing different doses of PRO to adult rats. Induction of steroidogenesis in the ovary's interstitial and thecal cells is impacted by alterations in the hypothalamic-pituitary-gonadal axis (Kakuta et al., 2012). The treated groups showed an increase in ovarian weight due to an increase in gonadotropin hormones (FSH and LH), which, in turn, resulted in structural and histological changes, including alterations in ovarian weight, diameter, and the number of ovarian follicles at each stage (Żukowska-Arendarczyk, 1981). The cells of atretic follicles and the theca interna are the sources of ovarian stromal-interstitial cells, which can generate andro-

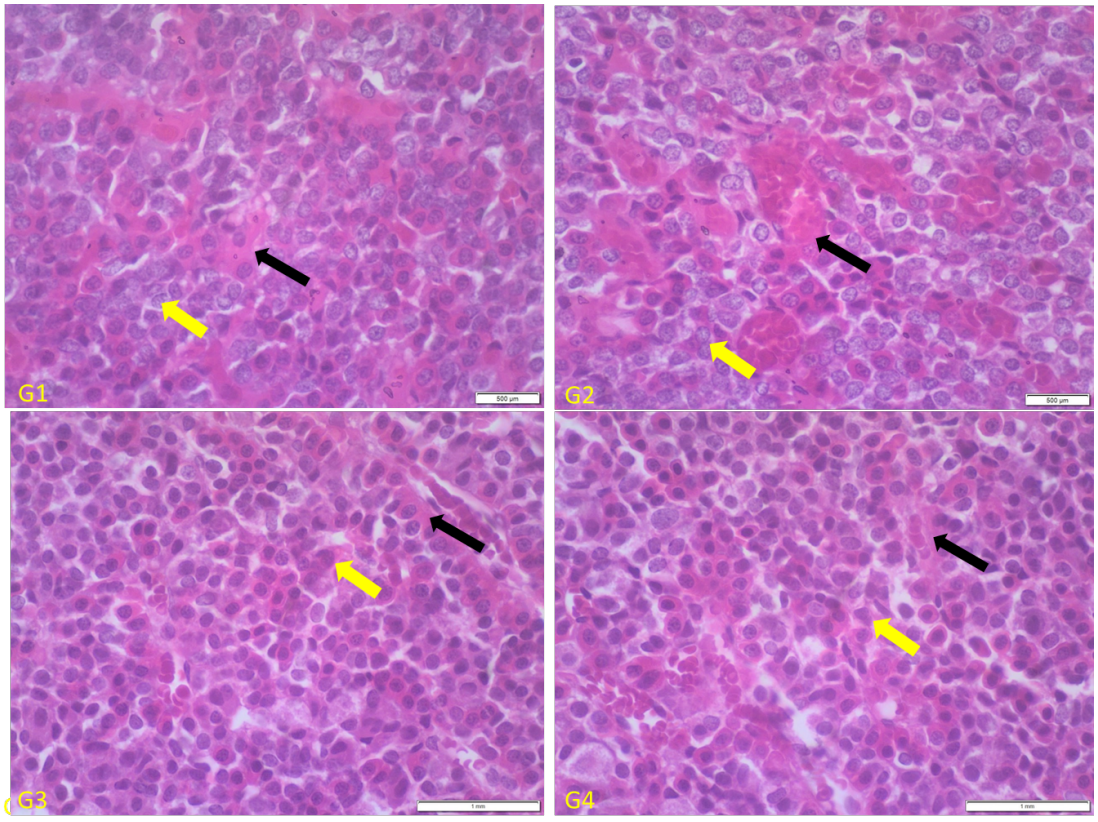

Figure 7. Light microscope images of female rat pituitary gland histological sections after various PRO supplement dosages
Note: Higher vascularization (black arrow) and more closely spaced active endocrine cells in the pars distalis (yellow arrow) are characteristics of the G4 group (H & E; ×40 magnification).

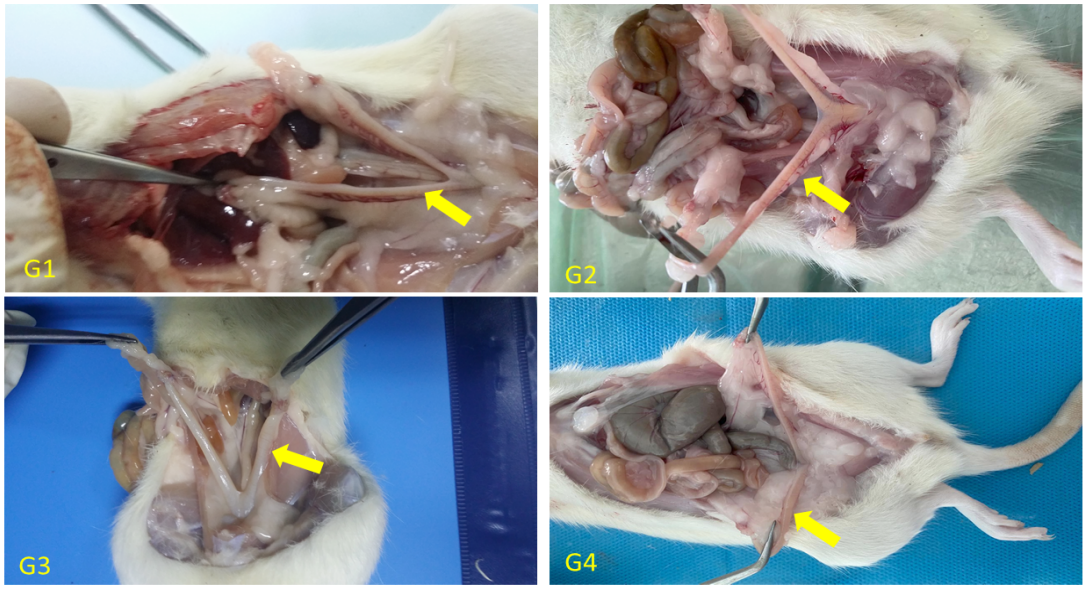

Figure 8. The morphology of the rats' uteri is of the duplex type

Note: The uterine horns from control and PRO-fed rats show normal and regular structure.

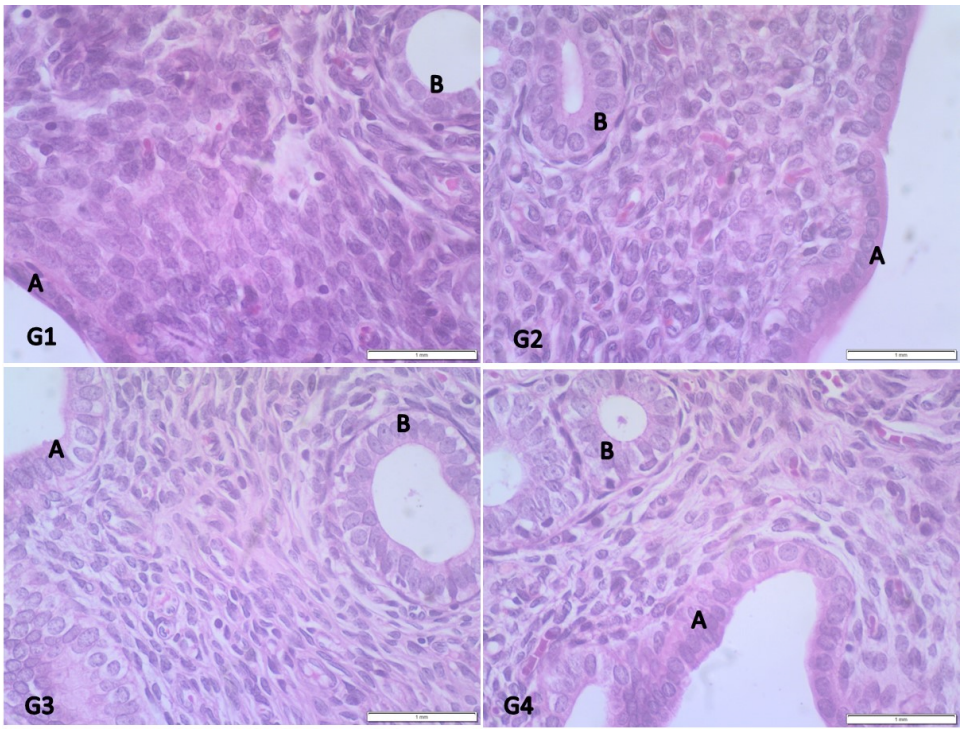

Figure 9. Photomicrographs of the rat uteri treated with different doses of PRO in the G1, G2, G3, and G4 experimental groups demonstrate that all groups' histological features are identical (H&E; ×40 magnification)

A) LE, B) GE.

Note: All groups showed normal architecture.

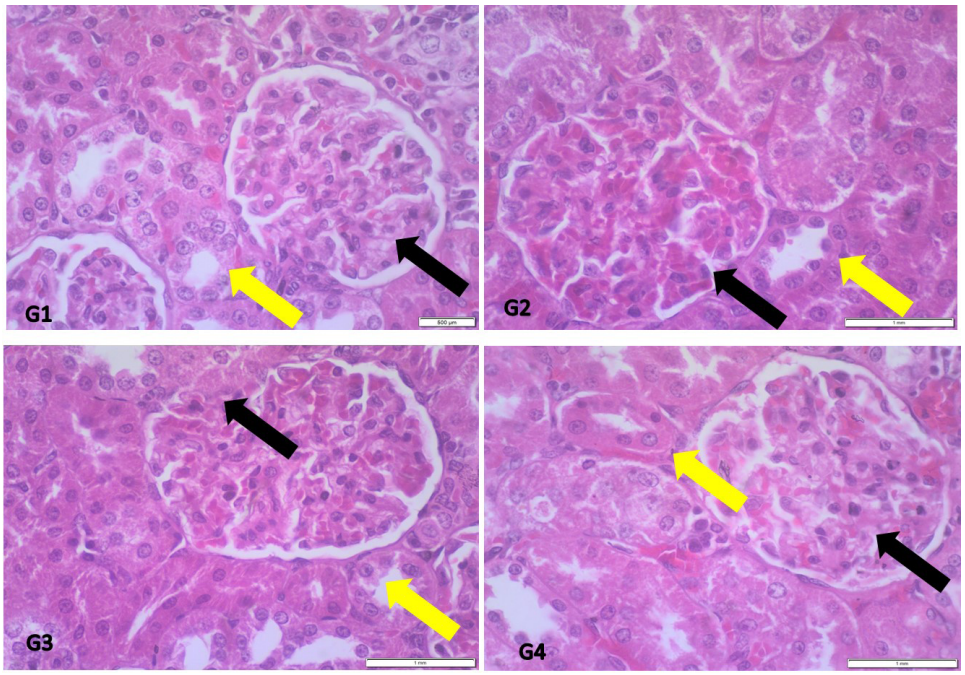

Figure 10. The photomicrograph displays the architecture of renal histology in rats 4 weeks after treatment with PRO

Note: All groups showed normal architecture. The groups that received PRO treatment are denoted by G1, G2, G3, and G4, with their corresponding graded concentrations of 0, 150, 300, and 500 mg/kg body weight per day.

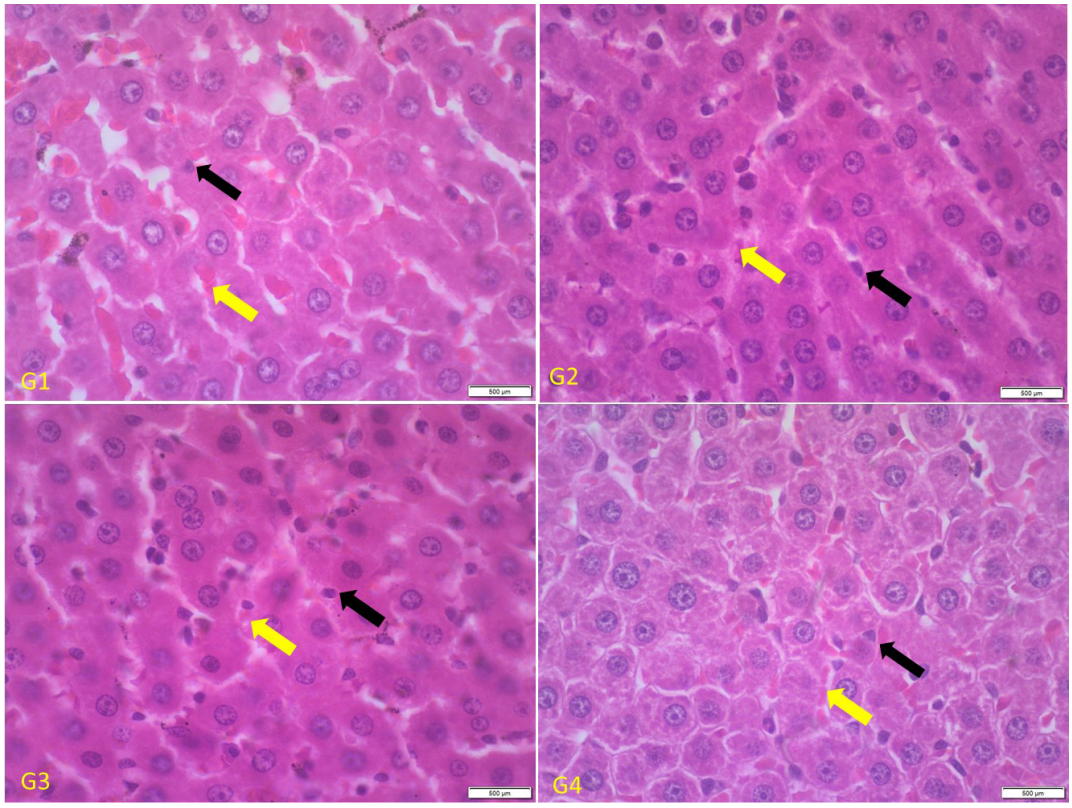

Figure 11. The photomicrograph shows the architecture of hepatic histology in rats in the 4th week after treatment with PRO
Note: All groups exhibited normal architecture. The groups that received PRO treatment are denoted by G1, G2, G3, and G4, with their corresponding graded concentrations of 0, 150, 300, and 500 mg/kg body weight per day.

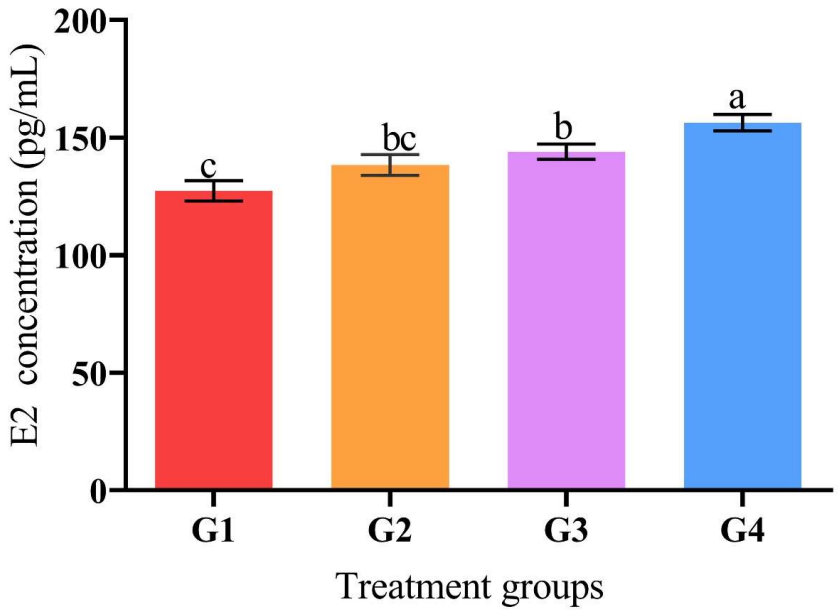

Figure 12. E2 serum concentrations in rats that were supplemented with different doses of PRO

Pg/mL: Picograms per milliliter.

Note: G4 had the greatest E2 level ($P<0.05$), and E2 concentrations increased significantly in a dose-dependent manner. The groups that received PRO treatment are denoted by G1, G2, G3, and G4, with their corresponding graded concentrations of 0, 150, 300, and 500 mg/kg body weight per day. At $P<0.05$, bars with distinct lettering denote statistical significance.

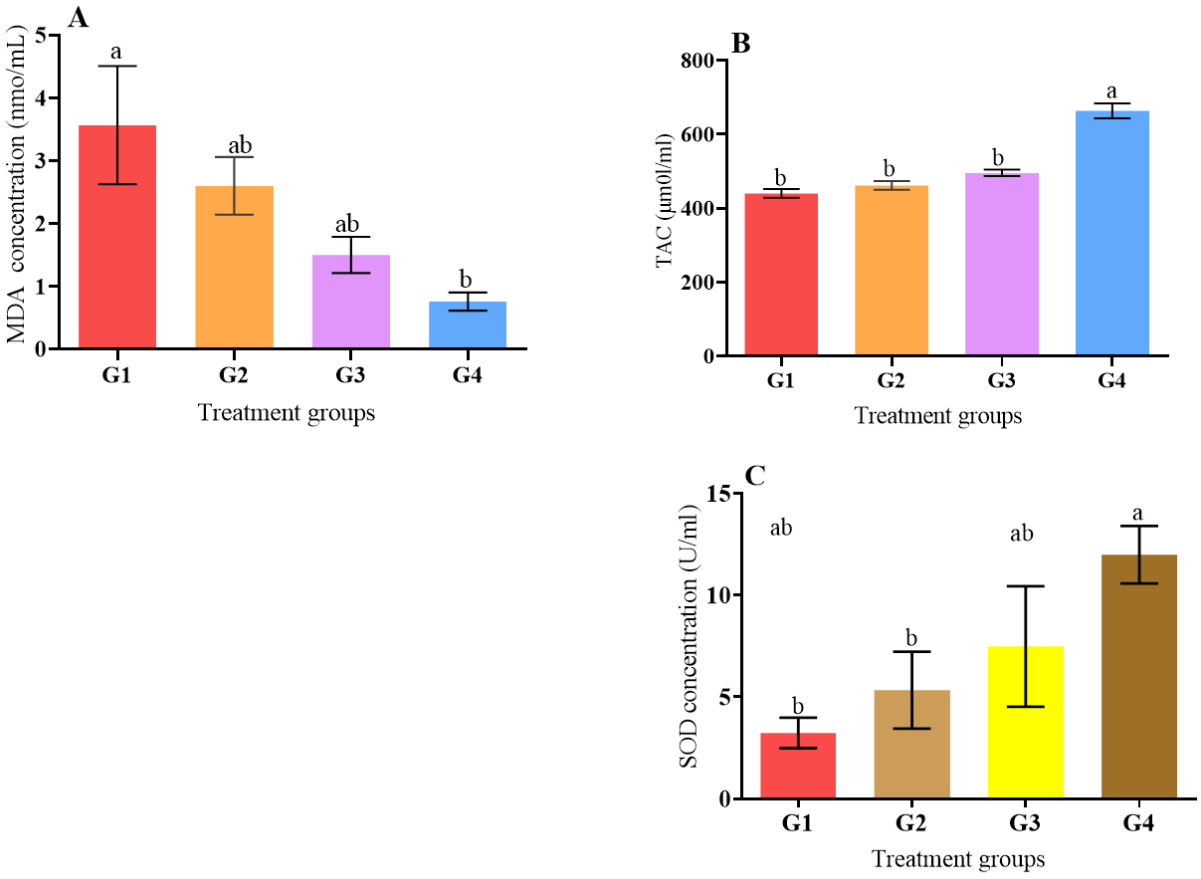

Figure 13. Impact of PRO on biomarkers of AO defense and oxidative stress in serum

Abbreviations: MDA: Malondialdehyde, TAC: Total antioxidant capacity, SOD: Superoxide dismutase, nmo/mL: Nanomoles per milliliter, µmol: Micromoles; U/mL: Units per milliliter.

Note: The groups that received PRO treatment are denoted by the letters G1, G2, G3, and G4, with their corresponding graded concentrations of 0, 150, 300, and 500 mg/kg body weight per day. At four weeks, there was a remarkably lower MDA concentration in G4 ($P<0.05$). G2 and G3 showed no significant alterations. TAC levels and SOD activity increased in the treatment groups in a dose-dependent manner, with the G4 group exhibiting higher levels of TAC and SOD ($P<0.05$). Means±SE are used to express data. A significant difference at $P<0.05$ is indicated by different letters a and b within the rows.

stenedione and testosterone (T) in response to the effect of LH (Drummond, 2006). Androgen promotes growth and development of surviving follicles and the corpus luteum (Speroff & Fritz, 2005). In addition, androgen is known to enhance the production of progesterone and E2 (Hillier & De zwart, 1981). Further studies on the impact of PRO supplements on the production of E2, a reproductive hormone, would complement the findings of the present study.

Electron microscopy and histochemistry studies revealed that OSE plays a major role in follicular rupture due to its content of lysosome-like inclusions, which produce proteolytic enzymes (Bjersing & Cajander, 1975). In addition to the secretion of the enzymes, OSE cells possess more receptors for agents that have differentiation-regulatory capabilities and promote growth (Auersperg et al., 2001).

Similarly, the action of insulin-like growth factor (IGF)-1 may be involved in the increased thickness of the ovarian epithelium (Ostrowska et al., 2001). Notably, Tamura et al. (2009) found a correlation between this growth factor and the production of estrogen and progesterone hormones. This is consistent with the findings of the earlier study, which showed thicker OSE and higher growth factor expression in ovarian epithelium and stromal cells (Albishtue et al., 2019b). These findings provide additional evidence of the effect of PRO on reproduction and fertility.

Table 1. Ovarian histomorphometric data of rats assessed at the pro-estrous phase

Parameters	Mean±SE			
	G1 Group	G2 Group	G3 Group	G4 Group
No. of interstitial cells	130.83±1.4^{c}	152.33±2.19^{bc}	180.5±4.31^{b}	180.17±6.51^{a}
Thickness of OSE (un)	4.83±0.42^{b}	6.98±0.4^{ab}	7.58±0.39^{ab}	9.48±0.28^{a}

OSE: Ovarian surface epithelium; Un=micro meter.

Note: Both the thickness of the OSE and the quantity of interstitial cells increased in a dose-dependent manner with prolonged PRO use. A significant difference at P<0.05 is shown by different letters a, b, and c within the rows. (×100 magnification).

Table 2. Histomorphometric data in the pars distalis of rats assessed at the pro-estrous phase

Parameters	G1 Group	G2 Group	G3 Group	G4 Group
No. of endocrine cells	90±0.93^{c}	135±2.03^{b}	166±2.44^{ab}	170±2.77^{a}
No. of blood vessels	15±1.32^{bc}	18±0.97^{c}	20±1.73^{b}	26±1.86^{a}

Note: The number of active gonadotropic cells and the degree of vascularization in the pars distalis were significantly higher (P<0.05) in the G4 group compared to the control group. A significant difference at P<0.05 is shown by different letters a, b, and c within the rows.

According to Sen et al. (2001), in human endometria, E2 regulates the production of the sialic acid binding protein. This study found that uterine weight and length in the treatment groups significantly increased in comparison to the control group. This finding supports the hypothesis that uterine weight and length are correlated with the estrogen concentration of PRO.

In the current study, PRO-induced dose-dependent alterations were seen in the histological analysis of the uteri of the various treatment groups. These changes were evidenced by the thickening of the endometrium, GE, and LE, as well as an increase in the number of uterine glands. The maintenance of the estrous cycle during the peri-implantation stage of conceptus growth and conceptus survival depends on the secretions of the endometrial glands (Gray et al., 2002). Thus, PRO increases uterine glandular secretions, including growth factors, such as epidermal growth factor and vascular endothelial growth factor, hormones, and transport proteins for conceptus development, which improves uterine physiology (Sen et al., 2001; Albishtue et al., 2019a).

Prior research indicates that the serum concentrations of glucose, creatinine, and urea were unaffected by PRO administration. On the other hand, in response to PRO supplementation, there were reduced (P<0.05) blood levels of cholesterol, total lipids, aspartate aminotransferase, and MDA. The serum concentrations of urea, creatinine, and glucose were not impacted by PRO administration, according to a prior study (Juwita et al., 2023; Sierra-Galicia et al., 2023). In the present study, no histological lesions were observed in the kidney or liver, while histomorphometric data revealed that the treated groups had fewer Kupffer cells in their livers. This finding is consistent with the fact that PRO helps regulate the function of the reproductive system by blocking inflammatory pathways that cause hypertension (Gulhan, 2019).

According to several studies, PRO has numerous essential properties, including antibacterial and anti-inflammatory effects. PRO improved growth performance, weight, immune functions, and AO status (Shedeed et al., 2019; Alwaeely et al., 2021; Bava et al., 2024). When rats were treated with PRO, their uterine cells and glands showed higher activity. Additionally, PRO stimulates the growth of uterine glands and has oestrogenic properties (Muhammad et al., 2013).

According to a prior study, oral PRO treatment dramatically increased the wet weight of the uterus and the thickness of the LE in ovariectomized rats compared to the equivalent values in the control group (Okamoto et al., 2015). This finding is consistent with our findings, which show that PRO increases the thickness of the uterine glands and the proliferation of LE, GE, and uterine glands.

Steroid hormones significantly affect the uterine structure via estrogen receptors (E2R) and progesterone receptors (P4R) (Albishtue et al., 2018a; Albishtue et al., 2019b). The proliferation of uterine epithelia is significantly influenced by estrogen, which stimulates P4R to

Table 3. Uterine histomorphometric data assessed in rats at the proestrus phase

Parameters	Mean±SE			
	G1 Group	G2 Group	G3 Group	G4 Group
Thickness of GE (un)	13.27±1.2^{bc}	15.84±1.5^{c}	23.88±0.9^{b}	32.52±1.8^{a}
Thickness of LE (un)	14.27±1.23 ^{bc}	18.84±1.47^{c}	22.88±0.9^{b}	30.52±1.77^{a}
Thickness of Endothelium (un)	277.38±18.3^{c}	325.63±17.37 ^{b}	396.74±17.89^{b}	583.9±24.33^{a}

Abbreviations: OES: Ovarian epithelium surface; LE: Luminal epithelium; GE: Glandular epithelium; Un=micro meter.

Note: PRO increased the thicknesses of the endometrium, GE, and LE in a dose-dependent manner. At P<0.05, the thicknesses of the endothelium, LE, and GE in all treatment groups were higher than those in the control group. All treated groups had significantly (P<0.05) greater LE, GE, and endothelium thicknesses than the controls. A significant difference at P<0.05 is shown by different letters a, b, and c within the rows.

Table 4. Hepatic histomorphometric parameters in the rats assessed at the pro-estrus stage

Parameters	Mean±SE			
	G1 Group	G2 Group	G3 Group	G4 Group
No. of Kupffer cells	65.5±1.48^{a}	60.67±1.36^{b}	56.5±1.63^{b}	45.33±1.76^{c}

Note: The G4 group had a significantly lower number of Kupffer cells than the G1 group (P<0.05). Different letters a, b, and c within the rows indicate a significant difference at P<0.05 (×1000 magnification).

improve progesterone's activity. The growth of uterine epithelia is significantly influenced by estrogen, which also increases the effect of progesterone via activating P4R. This study demonstrated the correlation between PRO supplementation and enhanced densities of stromal cells, GE, and LE. These results provide insight into the role and mechanism of action of PRO supplementation in improving reproduction.

Menstrual dysfunction rates, including amenorrhoea and irregular menstruation, have been linked to low estrogen levels in women (Bergemann et al., 2005). PRO has a high phytoestrogen content and can be used to treat gynecological diseases. Additionally, studies have demonstrated that oral PRO administration causes estrogenic activity in the body's organs that express estrogen receptors, making it a potentially effective treatment for menopausal symptoms (Okamoto et al., 2015). PRO's flavonoid components have similarities to a class of non-steroidal chemicals called selective estrogen receptor modulators, which interact with ERs and have different profiles unique to different tissues (Al-Qtaitat, 2014).

This study found a correlation between PRO and increased synthesis of reproductive hormones, suggesting that PRO has a significant impact on ovarian and uterine structures that has yet to be elucidated.

The positive effect of PRO on steroid hormone synthesis was demonstrated by the dose-dependent increase in serum E2 levels. This result is consistent with that of Zingue et al. (2017), who found that ovariectomized rats administered a PRO dietary supplement had higher serum E2 levels, suggesting that PRO can be utilized as estrogen treatment to address ovariectomized-induced aging. Guzeloglu-Kayisli et al. (2009) identified the uterus, mammary glands, and ovaries as target organs for progesterone and estrogen.

PRO has a high concentration of polyphenols, amino acids, polysaccharides, steroids, terpenoids, phenylpropanoids, aldehydes and ketones, and many other organic and inorganic compounds (Zulhendri et al., 2021).

In conjunction with gonadotropins, estrogens influence the growth and differentiation of granulosa cells. The production of more E2 is indirectly stimulated by neurons in the hypothalamus, which then secretes gonadotropin-releasing hormone.

This hormone activates the anterior pituitary's gonadotropic cells, leading to the release of LH and FSH into the bloodstream (Sarkar et al., 1976; Rajendren et al., 2001). FSH has multiple reproductive functions, including stimulating ovarian growth and promoting follicular devel-

opment. Conversely, LH regulates ovulation, follicular maturation, and the development of the corpus luteum, and intervenes in the synthesis of E2 and P4. The current research confirmed that PRO raises E2 concentrations, most likely in conjunction with an increase in LH release from the pituitary glands.

E2 has been demonstrated to be essential for female sexual desire and behavior in all female mammals. In contrast, testosterone and E2 are responsible for controlling women's libido. However, E2 appears to be the more prominent candidate for this function (Cappelletti & Wallen, 2016).

Additionally, the current study demonstrated that PRO enhances the vascularization of the pituitary gland and increases the number of gonadotropic cells that produce vital hormones. Therefore, the endogenous increase in production, which is linked to the proliferative impact of PRO on the pituitary gland, is most likely responsible for the rise in serum hormone levels.

PRO contains compounds such as caffeic acid and triterpenoids, which possess estrogenic properties (Zingue et al., 2017). According to a recent study on PRO in areas other than reproduction, it enhanced the production of steroid hormones, such E2 and acted as an AO (Kaya et al., 2022). PRO dramatically reduced MDA levels ($P<0.05$) in the G2, G3, and G4 groups in the current study compared to the G1 group.

However, only the G4 group showed a significant difference. In comparison to the control group, the AO status was higher ($P<0.05$) in the treatment groups. This result is consistent with a previous study where PRO supplementation demonstrated strong AO activity that reduces OSB levels (Kanazashi et al., 2023). In addition, the flavonoid and phenolic composition of PRO contributes to its AO activity and ability to scavenge free radicals, protecting lipids and vitamin C from oxidative damage (Seven et al., 2014; Kocot et al., 2018).

In conclusion, the current results suggest that PRO supplementation has a significant effect on the hypothalamus-pituitary-ovarian-uterine axis at multiple levels. The increased proliferation of ovarian and uterine structures, including GE, LE, and stromal cells, suggests that PRO may have the potential to improve ovarian and uterine functions, including reproduction.

Moreover, it decreases body weight and oxidative stress levels while enhancing immunity and libido by increasing serum levels of reproductive hormones.

Ethical Considerations

Compliance with ethical guidelines

Animals were treated humanely in accordance with Institutional Animal Care and Use Committee guidelines for animal care. This study was approved by the Faculty of Veterinary Medicine at the University of Kufa, Kufa, Iraq (Project approval number:13775; Dated 28/5/2024).

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Authors' contributions

Study design: Abdulla A. Albishtue; Investigations: Ahzan Kh. Abduiameer; Pro preparation: Wurood R. Hassen; Experiments: Abdulla A. Albishtue, Ahzan Kh. Abduiameer, Mustafa Ali Alahmer; Data analysis: Abdulla A. Albishtue, and Sameer Taklif; Writing: Abdulla A. Albishtue, Mustafa Ali Alahmer, and Mohammed Al-Mousaw; Final approval: All Authors.

Conflict of interest

The authors declared no conflicts of interest.

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