Effect of Propolis on Precocious Puberty in Female Rats

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Abstract

Introduction: Nutrition and exposure to various chemicals, including environmental pollution, insecticides, and plant phytoestrogens (having oestrogen-like effects), are environmental factors that affect puberty onset. We aimed to identify the effects of propolis on precocious puberty and the reproductive system in prepubertal female rats (ovary, endometrium, breast).

Methods: Thirty-four 25-day-old prepubertal female Sprague-Dawley rats were included in the study. Rats were randomly divided into the propolis (n 17) and control groups (n 17). The primary endpoint was the number of rats that developed vaginal opening (It's a sign of puberty) at 12-day follow-up. In addition, the effect of propolis on ovary, uterus and breast tissue was evaluated.

Results: Vaginal patency occurred earlier in the propolis group. At the same time, a greater number of rats developed vaginal opening. The number of ovarian follicles (in all follicles), endometrial thickness, and mammary gland secretory gland area were significantly higher in the propolis group than in the control group (p-values <0.001, <0.001, <0.001, respectively). In addition, Ki-67 activity in the endometrium, breast tissue and ovary was more intense in the propolis group compared to the control group (p-values <0.001, <0.001, <0.001, respectively).

Conclusion: Propolis triggers precocious puberty in female rats, possibly by interacting with the oestrogen receptor. The mechanism of action of propolis should be considered before prescribing it. In addition, further studies are needed to explore the mechanism of action of propolis and to determine the component of propolis that triggers puberty.

Introduction

Adolescence is the transition period from childhood to adulthood. It involves the development of secondary sexual characteristics and reproductive ability, and sexual maturation [1]. Even under similar living conditions, the timing of puberty varies significantly among individuals, suggesting that many factors affect the onset of puberty, such as genetic and environmental factors, socioeconomic status, stress, metabolic rate, bone maturation, and body fat ratio [2–4]. In addition, nutrition and exposure to various chemicals, including environmental pollution, insecticides, and plant phytoestrogens (having oestrogen-like effects), are environmental factors that affect puberty onset [2, 5–8]. Propolis is a product from Apis mellifera hives, containing plant resins, beeswax, and minor constituents, including pollen and minerals [9]. Propolis is very heterogeneous and the composition is dependent upon plant sources and/or types of bees [10]. Although propolis has estrogenic effects [11–13], to the best of our knowledge, no previous study has evaluated the relationship between propolis and puberty onset. We aimed to identify the effects of propolis on precocious puberty and the reproductive system in prepubertal female rats.

Materials And Methods

This study was approved by the Animal Experiments Local Ethics Committee, Sakarya University, Turkey (01.07.2020: decision number: 34). Thirty-four 25-day-old prepubertal female Sprague-Dawley rats were included in the study. The number of rats was determined using G Power analysis (95% confidence interval, 80% Power). Rats were randomly divided into the propolis (n 17) and control groups (n 17). The weight of the rats was recorded before the experiments. The rats were sedated using anaesthetic doses of ketamine and xylazine; blood was obtained from the rats to measure the levels of luteinizing hormone (LH), follicle stimulating hormone (FSH), oestradiol, and testosterone. Water-soluble propolis (1 cc at a dose of 200 mg/kg; based on other similar studies) was administered to the propolis group by gavage for 12 days (about 1 human year). Water-soluble propolis contains 10% pure propolis and had prepared using water and glycol solution. The content of propolis is presented in the supplementary table. The control group was administered 1 cc of water by gavage. The animals were provided food and water *ad libitum*. Vaginal openness was measured at baseline and then daily to determine the time of puberty onset. The number of rats attaining puberty after 12 days of treatment was recorded. After 12 days of treatment, the rats were weighed. Then, the rats were sedated with the appropriate anaesthetic dose, blood was obtained to measure the hormone levels, and the rats were sacrificed. Uterine, ovarian, and breast tissues were obtained for histopathological and immunohistochemical evaluation.

Histopathological and immunohistochemical evaluation

The tissues were fixed with 10% formalin solution for 48 h and dehydrated with 60%, 70%, 80%, 96%, and 100% alcohol. Then, the samples were passed through a xylol series to make the tissues transparent. The tissues were embedded in paraffin and cut using a microtome. The sections were stained with hematoxylin-eosin (HE) to observe the histological changes in the ovary, endometrium, and mammary gland tissues. Photographs were acquired under a light microscope (Olympus CX31-Japan). Ten sections (10 μm each) were obtained from each ovary to determine the effects of propolis on the number of follicles. Only follicles with oocyte nuclei were counted to determine the follicle count. Follicles were classified into five stages: primordial, primary, secondary, antral, and atretic follicles [14]. The automated image analysis software Image J® was used to measure endometrial thickness (μm). All slides were examined under the microscope at 100× magnification [15]. Mammary gland tissues were examined using a Nikon eclipse inverted microscope (Nikon Corp., Tokyo, Japan), and the area was calculated using the NIS-element imaging system from the same manufacturer. The ratio of the area of the secretory epithelium and fat cells to the area of the stroma was calculated (μm^2) [16]. Ki-67 staining was used to demonstrate tissue stimulation and proliferation in the endometrium, mammary glands, and ovaries. The 4-μm-thick tissue samples were cut from the paraffin-embedded blocks and deparaffinized using a decreasing alcohol series. Citrate buffer was heated in the microwave for 20 min. The endogenous peroxidase activity was blocked with 3% H_2O_2. The primary antibody used was anti-Kİ-67 (1/400 dilution, Genetex). The secondary antibody (UltraVisionLarge Volume DetectionSystem Anti-rabbit by LabVision, HRP) was used in accordance with the manufacturer's instructions. DAB has been used for immunohistochemical staining of secondary antibody-labeled proteins in tissues. Mayer's hematoxylin was used as a counterstain. The prepared slides were covered in mounting medium (Aqueous Mounting

Medium by ScyTek). Proliferative activity, as assessed by Ki-67 staining, was semi-quantitatively analysed (h-score) by selecting 10 random fields, and 100 epithelial cells were photographed in each area. The Ki-67 index was calculated as the percentage of positively stained cells among the total cells assessed [17, 18] In the immunohistochemical analysis, Ki-67 staining and cell division rates in the mammary glands, ovary, and endometrium were compared between the control and propolis groups.

Hormonal assessment

The rats were sacrificed and blood samples collected. When the specimens had completely clotted, they were centrifuged at 1500 *g* for 10 min. Serum fractions were collected and frozen at −40°C until further use. LH, FSH, testosterone, and oestradiol levels were determined using a double antibody enzyme-linked immunosorbent assay (YLBiont brand Sandwich ELISA; Shanghai YL Biotech Co., Ltd., Shanghai, China). Hormone specific monoclonal antibody coated wells. Streptavidin–HRP-conjugated antibodies were added to all wells, except the blank well, and the wells were incubated at 37°C for 60 min. After incubation, the wells were washed to remove unbound antibody. The specimens were incubated with chromogen at 37°C for 10 min to develop a blue colour. Stop solution was added to terminate the reaction, reflected by a change in the colour of the solution from blue to yellow. The intensity of the yellow colour was directly proportional to the analyte concentration. The colorimetric readings were performed using the inappropriate wavelength for the micro ELISA reader. A standard curve was generated to calculate the sample concentrations. The results and the measurement range were specified as Rat LH 0.1-38 mIU/ml, Rat FSH 0.2-60 mIU/ml, Rat testosterone 10-3000 ng/L, Rat oestradiol 3-900 ng/L respectively. The within-run and between-run CV% of the analytes were given as <10%.

Statistical analysis was performed using the Statistical Package for the Social Sciences, version 20.0 software (IBM Inc., Chicago, IL, USA). Numerical variables were summarized by mean±standard deviation as appropriate. Normality of the numerical variables was assessed with the Kolmogorov–Smirnov test. To compare independent groups, the number of rats in the groups had low, nonparametric tests, including the Mann-Whitney U test. A p-value less than 0.05 was considered statistically significant.

Results

Laboratory, histopathological, and immunohistochemical data from the rats are presented in Table 1 and 2. Histopathological and immunohistochemical images are shown in Figures 1 and 2, respectively. The control and propolis groups had similar initial ($p = 0.535$) and final weights ($p = 0.809$) and baseline levels of LH ($p = 0.241$), FSH ($p = 0.158$), testosterone ($p = 0.524$), and oestradiol ($p = 0.667$). On day 12, the oestradiol and testosterone levels were higher in the propolis than control group ($p = 0.021$ and $p = <0.001$, respectively). The testosterone level decreased from baseline to day 12 in the control group, whereas it increased in the propolis group. Although the oestradiol level decreased in both groups, the decrease was smaller in the propolis compared with the control group. Vaginal openness was observed in only two rats (both on day 12) during the 12-day follow-up in the control group, whereas it was observed in all rats in the propolis group (day 4: 9 rats; day 5: 8 rats). The number of ovarian follicles, endometrial

thickness, and mammary gland secretory area were significantly higher in the propolis than control group ($p = <0.001$, $p = <0.001$, $p = <0.001$, respectively). In addition, Ki-67 activity in the endometrium, breast, and ovarian tissues was greater in the propolis than control group ($p = <0.001$, $p = <0.001$, $p = <0.001$, respectively).

Discussion

Many factors affect the age of puberty onset, including nutrition and exposure to environmental pollution, insecticides, and plant phytoestrogens, which have oestrogen-like effects [2, 5–8] Propolis consists of many chemicals that vary depending on the type of plant the bees choose to collect pollen or nectar. Several studies have reported that some of these chemicals, such as flavonoids, coumaric acids, and caffeic acids, have oestrogen-like activity [11, 12]. Okamoto et al.[11] showed that propolis increased the uterine wet weight and endometrial thickness in ovariectomized rats and stimulated ductal cell proliferation in the mammary glands via the oestrogen receptor. In the present study, propolis increased the endometrial thickness and secretory area of adipose tissue in the mammary glands. Additionally, it increased the number of follicles in the ovaries and Ki-67 staining in the ovary, uterus, and breast tissues, suggesting increased cell proliferation.

In female rats, vaginal opening, the first external sign of ovarian activity, is considered a sign of puberty and occurs at approximately postnatal 35-37 seen in days. The estrous cycle can start right after vaginal opening or within a week [19, 20]. Our study showed that the vaginal opening developed significantly earlier in the propolis compared with the control group. Vaginal openness was observed in only two rats (both on day 12) during the 12-day follow-up in the control group, whereas it was observed in all rats in the propolis group (day 4: 9 rats; day 5: 8 rats). In addition, while none of the rats in the control group could enter the estrous cycle (in vaginal smear), all the rats in the propolis group were in the estrous cycle.

Although genistein is predominantly found in soy, it is also one of the main components of propolis and estrogenic effect has been shown in previous studies [21–24].

Chrysin, the flavone group found in propolis, was found to inhibit the aromatase enzyme in most *in vitro* studies, leading to reduced oestrogen production [25]. A human study found no increase in the urine testosterone level after chrysin administration, suggesting that aromatase was not inhibited [26]. In the present study, the testosterone level was higher in the propolis than control group (testosterone decreased in the control group but increased in the propolis group), suggesting that propolis inhibits aromatase. However, the oestradiol level was also significantly higher in the propolis group (both the control and propolis groups had a decreased oestradiol level, but the decrease was more significant in the control group). These changes may be due to the tremendous intra-cycle oestradiol change due to the high number of rats in the estrous cycle in the propolis group. For this reason, estradiol values can be very different according to the period of the rats in the propolis group (especially in the proestrus period). In fact, it is not correct to compare estradiol because we do not know exactly what stage of the estrous cycle the rats in the propolis group are in. At the end of the study, no difference was found in the

gonadotropin level between the propolis and control groups. Presumably, propolis triggers precocious puberty by interacting with the oestrogen receptor and oestradiol/testosterone ratio, rather than increasing the gonadotropin or oestradiol level. In addition, the steroid/oestrogen-like rings of some flavonoids/phenolics, which are abundant in propolis, may induce changes in the steroid pathway and trigger precocious puberty due to interaction with the oestrogen receptor. Contrary to our study, some studies have shown that polyphenols in green tea prevent prepubertal puberty [27, 28]. This opposite effect of green tea or propolis may be due to the different ratios of polyphenols in the food used. (While the rate of catechin was high in green tea, the rate of Chrysin and Caffeic acid phenethyl ester was higher in our propolis).

Strengths/Limitations

Our study is the first study evaluating the relationship between propolis and puberty, as far as we know. Clinical findings and histological findings were supported by immunohistochemical staining. The small sample size limits our results. To account for this, conservative statistical methods, including nonparametric tests, were employed to mitigate the risk of type I error.

Conclusion

Propolis triggers precocious puberty in female rats, possibly by interacting with the oestrogen receptor. The mechanism of action of propolis should be considered before prescribing it. In addition, further studies are needed to explore the mechanism of action of propolis and to determine the component of propolis that triggers puberty.

Declarations

Financial Disclosure:

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Financial interests:

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions:

R.P, E.C and O.B. designed the study, collected samples, and performed analysis of the data. R.P, E.C and F.B.T. data processing, and interpretation. E.C. and F.B.T. performed statistical analysis and data interpretation. E.C and F.B.T. performed sex hormone analysis and data interpretation. R.P, F.B.T and O.B. took part in sample collection and data interpretation. Ö.B performed histolochemical analysis. All authors took part in manuscript preparation and revised and approved the final version of the text.

Ethics Consent:

This study was approved by the Animal Experiments Local Ethics Committee. Sakarya University, Turkey (01.07.2020: decision number: 34).

Data Availability:

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/yFiRRO

Conflict of interests:

The authors declares that there are no conflict of interests.

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Tables

Due to technical limitations, table 1 and 2 PDFs are only available as a download in the Supplemental Files section.

Figures

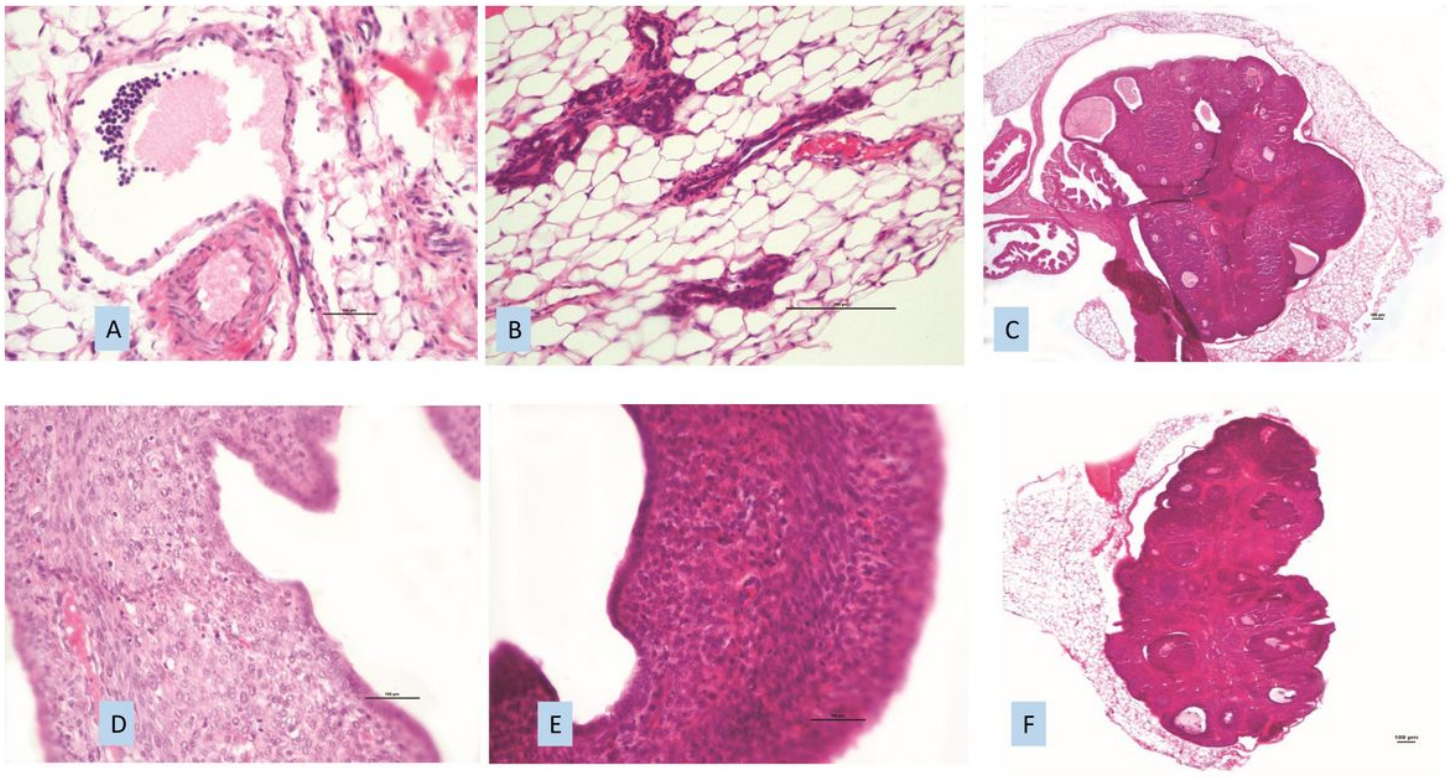

Figure 1

Larger secretory areas (active state) were observed in the adipose tissue of the mammary glands in the propolis group (A) than control group (B). In ovarian tissue, there were more secondary, antral, and corpus luteum follicles in the propolis group (C) than control group (F). The endometrial layer was thicker in the propolis group (E) than control group (D). H.E pictures. 40x lens, 100 scale bar.

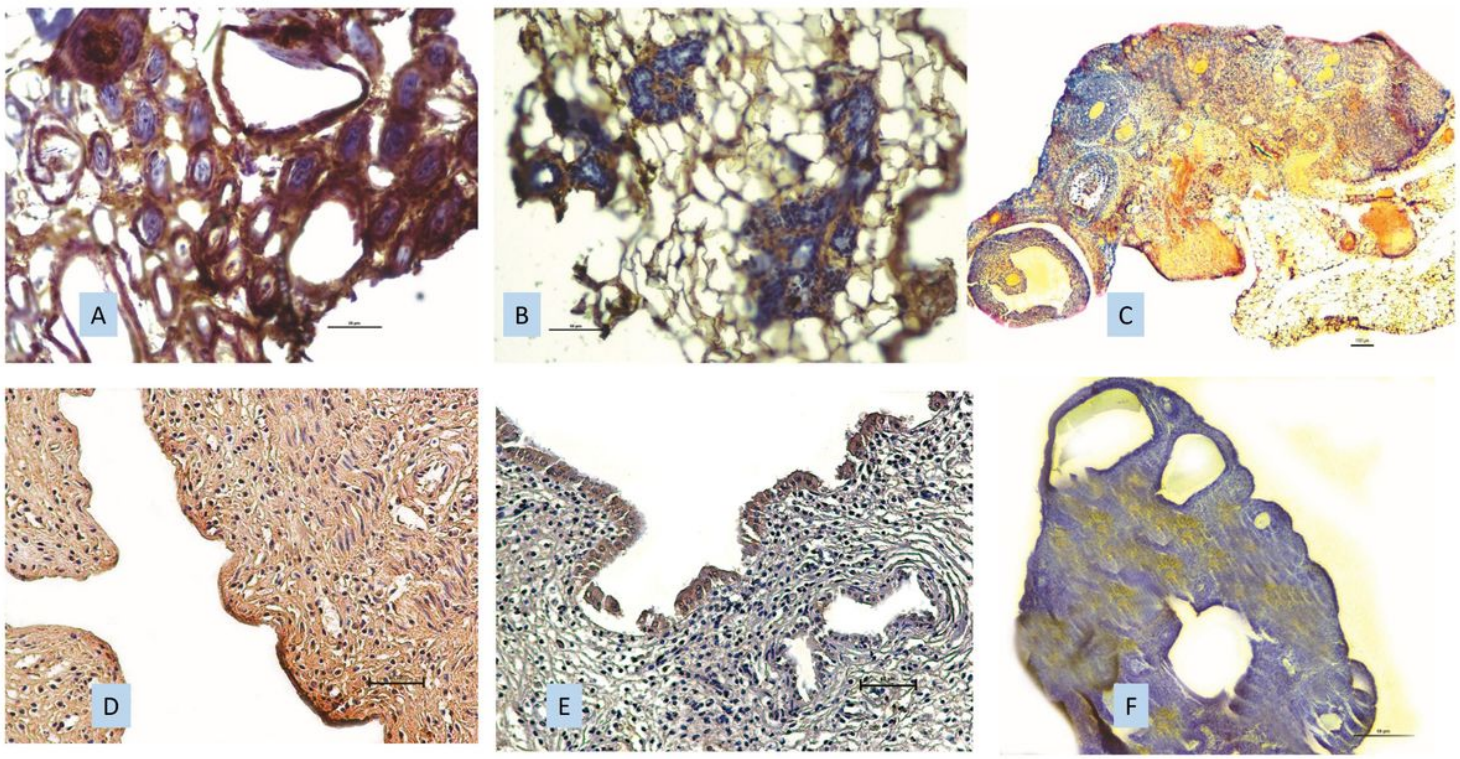

Figure 2

Due to cell development and proliferation, Ki-67 staining intensity was greater in the mammary glands, ovary, and endometrium (due to thickening) in the propolis group (A, C, and D, respectively) compared with the control group (B, E, and F, respectively). Ki-67 immunoreactivity preparations. 200x lens, 100 scale bar.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytable1.pdf
- Table1.pdf
- Table2.pdf