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 (µm²) [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% H₂O₂. The primary antibody used was anti-Ki-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 \(-40^\circ C\) 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 chrysian 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:

This project was funded by Sakarya University Scientific Research Project Coordinator.

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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**Conflict of interests:**

The authors declares that there are no conflict of interests.

**References**

1. Garibaldi LR, Chemaitilly W (2020). Disorders of Pubertal Development. In: Kliegman RM, St Geme JW, Blum NJ, Shah SS, Tasker RC, Wilson KM (eds) Nelson Textbook of Pediatrics. 21st ed. Elsevier - Health Sciences Division, Philadelphia, pp 2899-2912

2. Finlayson C, Styne DM, J. Jameson JL (2016) Endocrinology of Sexual Maturation and Puberty. In: Jameson JL, De Groot LJ, de Kretser DM, Giudice LC, Grossman A, Melmed S, Potts JT, Weir GC (eds) Endocrinology: Adult and Pediatric. 7th ed. Elsevier - Health Sciences Division, Philadelphia, pp 2119-2129

3. Macedo DB, Silveira LF, Bessa DS, Brito VN, Latronico AC (2016) Sexual Precocity—Genetic Bases of Central Precocious Puberty and Autonomous Gonadal Activation Endocr Dev 29:50-71. [https://doi.org/10.1159/000438874](https://doi.org/10.1159/000438874)

4. Christoforidis A, Skordin N, Fanis P, et al (2017) A novel MKRN3 nonsense mutation causing familial central precocious puberty. Endocrine 56:446-449. [https://doi.org/10.1007/s12020-017-1232-6](https://doi.org/10.1007/s12020-017-1232-6)

5. Euling SY, Selevan SG, Pescovitz OH (2008) Role of environmental factors in the timing of puberty.” Pediatrics vol. 121 Suppl 3: S167-71. [https://doi.org/10.1542/peds.2007-1813C](https://doi.org/10.1542/peds.2007-1813C).

6. Parent AS, Teilmann G, Juul A, Skakkebaek NE, Toppari J, Bourguignon JP (2003) The timing of normal puberty and the age limits of sexual precocity: variations around the world, secular trends, and changes after migration. Endocr Rev 24:668-693. [https://doi.org/10.1210/er.2002-0019](https://doi.org/10.1210/er.2002-0019)
7. Tassinari R, Mancini FR, Mantovani A, Busani L, Maranghi F (2015) Pilot study on the dietary habits and lifestyles of girls with idiopathic precocious puberty from the city of Rome: potential impact of exposure to flame retardant polybrominated diphenyl ethers. J Pediatr Endocrinol Metab 28:1369-1372. https://doi.org/10.1515/jpem-2015-0116

8. Acerini CL, Hughes IA (2006) Endocrine disrupting chemicals: a new and emerging public health problem?. Arch Dis Child 91:633-641. https://doi.org/10.1136/adc.2005.088500

9. E. L. Ghisalberti (1979) Propolis: A Review. Bee World 60:59-84. https://doi.org/10.1080/0005772X.1979.11097738

10. Salatino A, Salatino M.L.F (2021) Scientific note: often quoted, but not factual data about propolis composition. Apidologie 52:312–314. https://doi.org/10.1007/s13592-020-00821-x

11. Okamoto Y, Tobe T, Ueda K, Takada T, Kojima N (2015) Oral administration of Brazilian propolis exerts estrogenic effect in ovariectomized rats. J Toxicol Sci 40:235-242. https://doi.org/10.2131/jts.40.235

12. Song YS, Jin C, Jung KJ, Park EH (2002) Estrogenic effects of ethanol and ether extracts of propolis. J Ethnopharmacol 82:89-95. https://doi.org/10.1016/s0378-8741(02)00159-9

13. Jung BI, Kim MS, Kim HA, et al (2010) Caffeic acid phenethyl ester, a component of beehive propolis, is a novel selective estrogen receptor modulator. Phytother Res 24:295-300. https://doi.org/10.1002/ptr.2966

14. Ozcan P, Takmaz T, Tok OE, Islek S, Yigit EN, Ficicioglu C (2020) The protective effect of platelet-rich plasma administrated on ovarian function in female rats with Cy-induced ovarian damage. J Assist Reprod Genet 37:865-873. https://doi.org/10.1007/s10815-020-01689-7

15. Zingue S, Nde CBM, Michel T, et al (2017) Ethanol-extracted Cameroonian propolis exerts estrogenic effects and alleviates hot flushes in ovariectomized Wistar rats. BMC Complement Altern Med 17:65. https://doi.org/10.1186/s12906-017-1568-8

16. Leonel ECR, Falleiros LR Junior, Campos SGP, Taboga SR (2017) Histological and immunohistochemical characterization of the Mongolian gerbil's mammary gland during gestation, lactation and involution. Acta Histochem 119:273-283. https://doi.org/10.1016/j.acthis.2017.02.003

17. Yokoyama Y, Takahashi Y, Morishita S, Hashimoto M, Niwa K, Tamaya T (1998) Telomerase activity in the human endometrium throughout the menstrual cycle. Mol Hum Reprod 4:173-177. https://doi.org/10.1093/molehr/4.2.173

18. McCampbell AS, Walker CL, Broaddus RR, Cook JD, Davies PJ (2008) Developmental reprogramming of IGF signaling and susceptibility to endometrial hyperplasia in the rat. Lab Invest 88:615-626. https://doi.org/10.1038/labinvest.2008.29

19. Holder MK, Blaustein JD (2014) Puberty and adolescence as a time of vulnerability to stressors that alter neurobehavioral processes. Front Neuroendocrinol 35:89-110. https://doi.org/10.1016/j.yfrne.2013.10.004

20. Terasawa E, Fernandez DL (2001) Neurobiological mechanisms of the onset of puberty in primates. Endocr Rev 22:111-151. https://doi.org/10.1210/edrv.22.1.0418
21. Coskun İ, Duymaz GM, Dastan T, et all (2018) The Characterization and Bioactive Composition of Turkish Propolis. J.Apit.Nat 1:39-39. https://dergipark.org.tr/tr/pub/jan/issue/40577/489438
22. Volpi N, Bergonzini G (2006) Analysis of flavonoids from propolis by on-line HPLC-electrospray mass spectrometry. J Pharm Biomed Anal 42:354-361. https://doi.org/10.1016/j.jpba.2006.04.017
23. National Toxicology Program (2008) Toxicology and carcinogenesis studies of genistein (Cas No. 446-72-0) in Sprague-Dawley rats (feed study). Natl Toxicol Program Tech Rep Ser 545:1-240. https://ntp.niehs.nih.gov/ntp/htdocs/lt_rpts/tr545.pdf
24. Al-Nakkash L, Markus B, Batia L, Prozialeck WC, Broderick TL (2010) Genistein induces estrogen-like effects in ovariectomized rats but fails to increase cardiac GLUT4 and oxidative stress. J Med Food 13:1369-1375. https://doi.org/10.1089/jmf.2009.0271
25. Balam FH, Ahmadi ZS, Ghorbani A (2020) Inhibitory effect of chrysin on estrogen biosynthesis by suppression of enzyme aromatase (CYP19): A systematic review. Heliyon 6:e03557. https://doi.org/10.1016/j.heliyon.2020.e03557
26. Gambelunghe C, Rossi R, Sommavilla M, et al (2003) Effects of chrysin on urinary testosterone levels in human males. J Med Food 6:387-390. https://doi.org/10.1089/109662003772519967
27. Wu Y, Wang J, Cai W, Shen X (2016) Could tea polyphenols be beneficial for preventing the precocious puberty?. Med Hypotheses 95:24-26. https://doi.org/10.1016/j.mehy.2016.07.017
28. Xie L, Tang Q, Yao D, et al (2021) Effect of Decaffeinated Green Tea Polyphenols on Body Fat and Precocious Puberty in Obese Girls: A Randomized Controlled Trial. Front Endocrinol (Lausanne) 12:736724. https://doi.org/10.3389/fendo.2021.736724

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

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- SupplementaryTable1.pdf
- Table1.pdf
- Table2.pdf