CHRYSIN INCREASED PROGESTERONE AND LH LEVELS, ESTROUS PHASE DURATION AND ALTERED UTERINE HISTOLOGY WITHOUT AFFECTING AROMATASE EXPRESSION IN RAT OVARY

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ABSTRACT

The effect of chrysin, a flavonoid found in *Passiflora spp*., honey and propolis on female sex hormones and on ovarian and uterine histology was studied. Treatment of Wistar rats with chrysin (100 mg/kg) for 28 days resulted in persistent or prolonged estrous phase. Histologically, no difference was found between different groups in the percentage of atretic preantral and antral follicles. Uteri of chrysin-treated groups had slit-like lumens during the estrous stage compared to the control group that had fluid-filled wide lumens. The height of luminal epithelium was higher in chrysin-treated groups and had more apoptotic cells. Immunohistochemical studies showed that chrysin had no effect on aromatase expression in the ovary or progesterone and estrogen-receptor alpha expression in the uterus. LH and progesterone, but not estrogen and FSH, levels were higher in chrysin-treated group. In conclusion, chrysin increased LH and progesterone levels and produced histological alterations in the uterus.

Keywords: Chrysin, Estrous, LH, Ovarian follicle, Progesterone, Uterus

INTRODUCTION

Chrysin (5,7-dihydroxyflavone) is a naturally occurring flavone-type flavonoid found in plants such as *Passiflora caerulea*, *P. incarnata* and *Oroxylum indicum* (Thakur, Mandal, & Banerjee, 2016) and in high quantities in honey and propolis (Mani & Natesan, 2018). Many research articles have reported potential benefits of chrysin when administered to laboratory animals. Such biological activities include antinociceptive (Farkhondeh, Samarghandian, Azimin-Nezhad, & Samini, 2015), anxiolytic (Rodríguez-Landa et al., 2019), anticonvulsant (Medina et al., 1998), antioxidant (Veerappan & Senthilkumar, 2015), antiangiogenic (Song, Kim, Lee, Kim, & Lee, 2016) and antihypertensive effects studied in rats (Veerappan & Senthilkumar, 2015). In addition to anti-inflammatory activity studied in guinea pigs and nude mice (Kaidama & Gacche, 2015; Ramírez-Espinosa et al., 2018). Furthermore, chrysin exhibited anti-allergic effect in BALB/c mice (Yao et al., 2016), hypolipidemic effect in C57BL/6 mice (Zarzecki et al., 2014) and anticancer activity *in-vitro* (Xia et al., 2015). Also, it exerted a protective effect against ischemia–reperfusion injury of ovaries (Melekoglu, Ciftci, Eraslan, Alan, & Basak, 2018) and radiation-induced premature ovarian failure in rats (Mantawy, Said, & Abdel-Aziz, 2019). Chrysin displayed beneficial actions on male reproductive system. Moreover, in adult rats it increased sperm motility, concentration, testosterone level, as well as glutathione, catalase and superoxide dismutase activity in testis (Ciftci, Ozdemir, Aydin, & Beytur, 2012). The

testosterone-boosting effect of chrysin was attributed to its role as a potent aromatase inhibitor that blocks the conversion of testosterone to estrogen (Kellis & Vickery, 1984). If testosterone is not converted into estrogen in male brain, negative feedback will not occur. Therefore, aromatase enzyme inhibition may result in increasing serum testosterone levels and result in improving male fertility (Ciftci et al., 2012). The effect of chrysin on female reproduction is poorly studied. Furthermore, the available literature focused on the anti-oxidant effect of chrysin on ovary and its protective effect on ischemia–reperfusion injury in rat ovaries (Melekoglu et al., 2018). Chrysin has been proved to have anti-cancer effect, anti-oxidant effect, potential use for neurological disorders, and boosting male fertility (Ciftci et al., 2012; Melekoglu et al., 2018; Mishra et al., 2021; Talebi et al., 2021). However, its effects on female reproduction, especially chrysin effect on uterus, is poorly investigated and need further

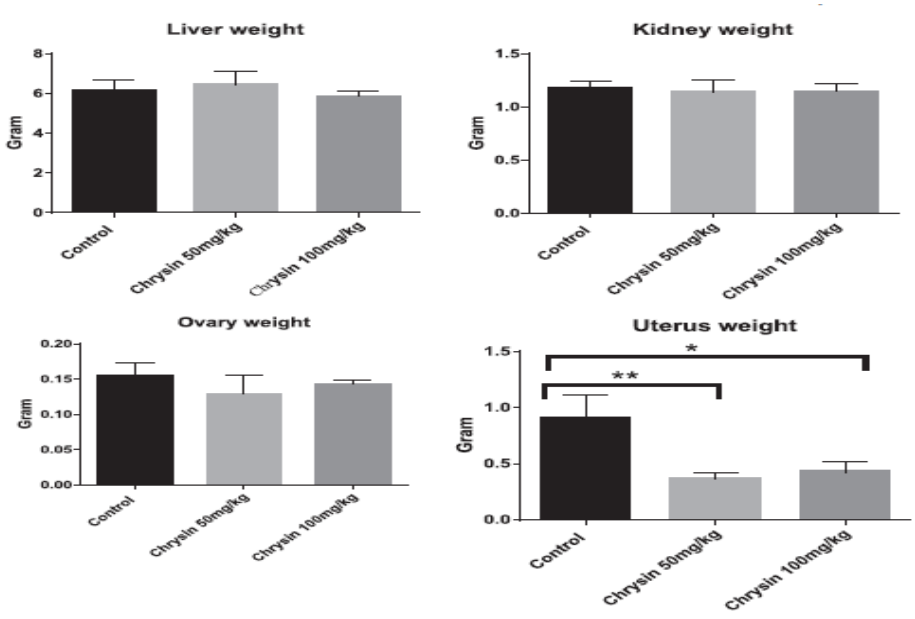

Fig. 1. Organ's weight in the control and chrysin-treated groups. * $p < 0.05$. ** $p < 0.01$.

investigation. Therefore, this work was designed to investigate the effect of chrysin on female hormone levels as well as on ovarian and uterine histology in rats. Furthermore, chrysin effect on aromatase enzyme expression in ovary and estrogen and progesterone receptor expression in uterus was investigated.

MATERIALS AND METHODS

Drugs, chemicals and kits

Chrysin (purity 97%) and carboxymethyl cellulose (CMC) were obtained from Sigma-Aldrich (USA). Elisa kits for the determination of rat luteinizing hormone (LH), rat follicle-stimulating hormone (FSH), rat estrogen and progesterone were obtained from Bluegene (China).

Experimental design

Twenty four, sexually mature, virgin female Wistar rats weighing 150–160 gm were purchased from the Jordan University of Science and Technology, Irbid, Jordan. Animals were acclimatized to the conditions in the animal room for 2 weeks (temperature 23 ± 2 ◦C, 12hr dark/12hr light). Vaginal smears were taken and checked daily between 12.00 and 14.00p.m to determine which stage of the estrous cycle the rats were in. Only female rats with regular 4–5 days estrous cycles were used in the study. Chrysin was injected i.p after suspending it in 1% CMC as previously published (Yao et al., 2016). The i.p route was chosen in this study due to poor intestinal absorption of chrysin (Walle, Otake, Brubaker, Walle, & Halushka, 2001). All animal experiments were approved by the ethical committee for scientific research at Al-Ahliyya Amman University (Ethical approval number: 1/4/2019–2020). Rats were randomly divided into 3 groups (8 rats each). The control group was administered a daily i.p injection of the vehicle (1% CMC). Groups 2 and 3 were administered a daily dose of 50 and 100 mg/kg chrysin, respectively for a treatment period lasting for 28 ± 2 days. The choice of doses was based on previous studies (Sirovina et al., 2013). For each female rat, treatment started and ended during the estrous phase of the estrous cycle. At the day of sacrifice, blood withdrawal was performed at 9–10 a.m. Blood samples were centrifuged at 3000 rpm for 10 min. The obtained serum was stored at □ 80 ˚C until hormone analysis was performed. Ovary, liver and kidney weights were recorded using a 6 digit-sensitive balance (Shimadzu, Japan) as well as uterus weight, including its fluid content. The ovaries and uteri were then transferred to 10% buffered formalin solution for the preparation of histological slides.

Histopathological, morphometric and immunohistochemical studies

Formalin-fixed, paraffin-wax embedded serial sections (4 μm thick) of ovaries and uteri were stained with hematoxylin and eosin (H & E). Then, sections were examined using Leica microscope and

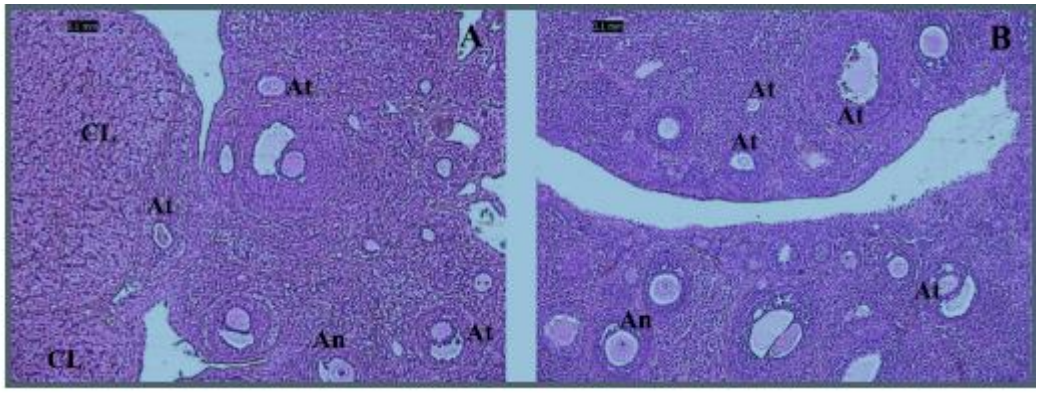

Fig. 2. Histology of rat ovary at the estrous stage. (A) Control, (B) Chrysin 100 mg/kg. An: antral follicle; At: atretic; CL: corpus luteum (H&E stain). Scale Barr = 100 μm.

software. Healthy and atretic antral follicles were counted blindly by 2 histologists separately at a total magnification of 40× while preantral follicles were counted at a total magnification of 100×. Classification of follicles was based on morphology and size (Mescher, 2018). The percentage of atretic follicles was calculated by dividing the number of atretic follicles by the total number of follicles in the whole section. Morphometric analyses of uterus were performed on mid-horn serial cross sections.

The height of luminal epithelium was quantified as previously mentioned (Amira, Zuki, Goh, & Noordin, 2011), (Vasilenko, Mead, & Weidmann, 1986). Immunohistochemical (IHC) staining was performed using Ventana Medical System automated stainer (USA) at The Specialty Hospital, Amman, Jordan. Ventana ultraview universal 3,3′ -diaminobenzidine (DAB) detection kit was used. Briefly, formalin-fixed, paraffin-embedded tissues were cut at 4 µm, mounted on positively charged slides (Surgipath Apex, Leica) and dried in an oven at 90 ◦C for 15 min. Following deparaffinization with Ventana EZ Prep solution, antigen retrieval was performed using Ventana Tris-based buffer solution (cell conditioning solution, CC1) at 95–100 ◦C for 60 min. Endogenous peroxidase was blocked with ultraview DAB inhibitor (3% H2O2). The slides were rinsed with Ventana reaction buffer between steps. Then, uterus sections were incubated at 37 ◦C for 60 min with mouse monoclonal primary antibody for estrogen receptor ER alpha/NR3A1 Antibody (33) (NB300-560), Novus Biologicals CO, USA (1:20 dilution); or rabbit monoclonal antibody to progesterone receptor (Rb mAb SP2 Abcam; 1: 200 dilution). The slides containing ovary sections were incubated with rabbit polyclonal anti-P450 aromatase (NB100-1596, Novus Biologicals NB100-1596; 1: 100 dilution). Slides were then incubated with ultraview universal horseradish peroxidase (HRP) multimer (<50 ug/mL). Visualization was achieved using ultraview universal 0.2% DAB chromogen with ultraview universal DAB H2O2 (0.04% H2O2), followed by enhancement with ultraview universal DAB copper (CuSO4 5 g/L). Slides were then counterstained for 6 min with hematoxylin and bluing reagent (0.1 M Li2CO3, 0.5 M Na2CO3). Each step of the immunostaining procedure was optimized and adjusted automatically with Benchmark IHC/ISH staining module (Ventana Medical Systems). For each section, four random fields were captured at 100x magnification using Leica digital camera connected to the light microscope (Leica Microsystems, Germany) and Leica application suite LAS EZ software version 1.8.0 (Leica Microsystems, Switzerland). The staining intensity of each IHC image was analyzed using Image J sofware (National Institutes of Health, USA).

Hormone analysis

Determination of serum LH, FSH, estrogen and progesterone level was performed using ELISA kits specific for rat (Bluegene, China) as per manufacturer directions.

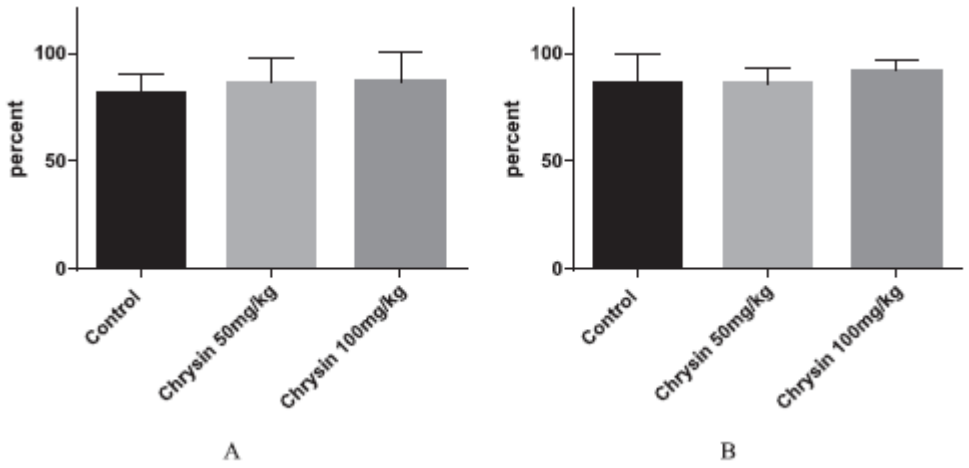

Fig. 3. (A) Percentage of atretic antral follicles in chrysin treated rats compared to vehicle-treated control group rats. (B) Percentage of atretic preantral follicles in chrysin treated rats compared to vehicle-treated control group rats.

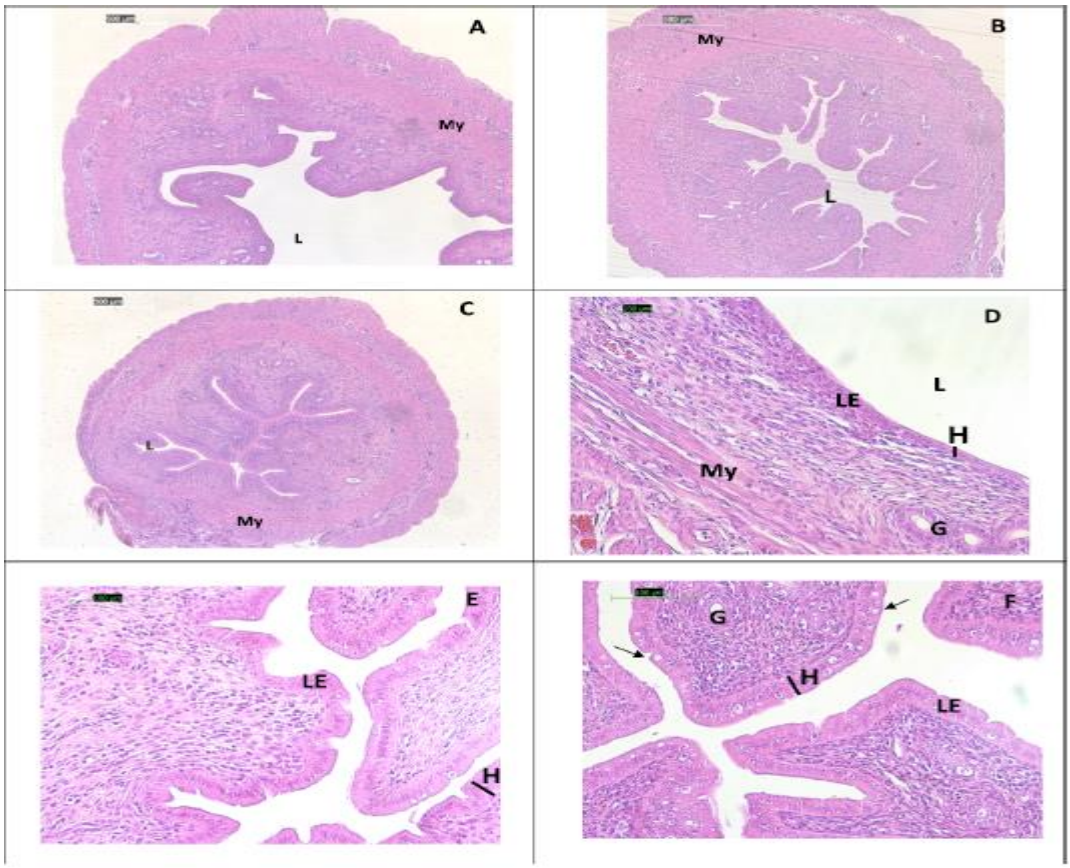

Fig. 4. Histological sections of uterus. (A) Uterus of a control group rat. (B) Uterus of a rat treated with 50 mg/kg chrysin. (C) Uterus of a rat treated with 100 mg/kg chrysin. Note slit-like lumen in chrysin-treated rats (B, C) and wide lumen in control (A). (D) Enlargement of uterus wall of a control-group rat. (E) Enlargement of uterus wall of a 50 mg/kg chrysin-treated rat. (F) Enlargement of uterus wall of a 100 mg/kg chrysin-treated rat. Apoptosis was more evident in luminal epithelium (arrows) in 100 mg/kg chrysin treated group. G: uterine gland; H: height of epithelium. L: lumen; LE: luminal epithelium; My: myometriun. Scale Barr = 500 μm in figure A,B and C. Scale Barr = 100 μm for figure D,E and F (H&E stain).

2.5. Statistical analysis

GraphPad Prism 6 was used to analyze data. All parameters were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc. Values were represented as mean ± standard error of the mean (SEM). *P* value ≤ 0.05 was considered statistically significant.

RESULTS

No signs of toxicity or mortality were observed during the experiment and none of the rats was excluded from the study. Daily vaginal smear examination revealed regular estrous cycle in rats belonging to the control and chrysin 50 mg/kg groups. However, in chrysin 100 mg/ kg treated group, estrous phase lasted for more than 3 consecutive

days in 60% of the females. This effect was observed after 3 weeks from the beginning of treatment. Organs' weights at the time of sacrifice are illustrated in Fig. 1. In the control, but not in chrysin-treated groups, uteri were fluid-filled. The average weight of uterus, including their fluid content, in control group was significantly higher than in chrysin-treated groups (Fig. 1). No statistically significant difference in liver, kidney or ovarian weight was observed between different groups (Fig. 1). H&E-stained ovarian sections had similar morphology in the control and chrysin-treated groups as seen under the light microscope (Fig. 2). Morphometric studies of ovaries failed to find any statistically significant difference between different groups in the percentage of atretic

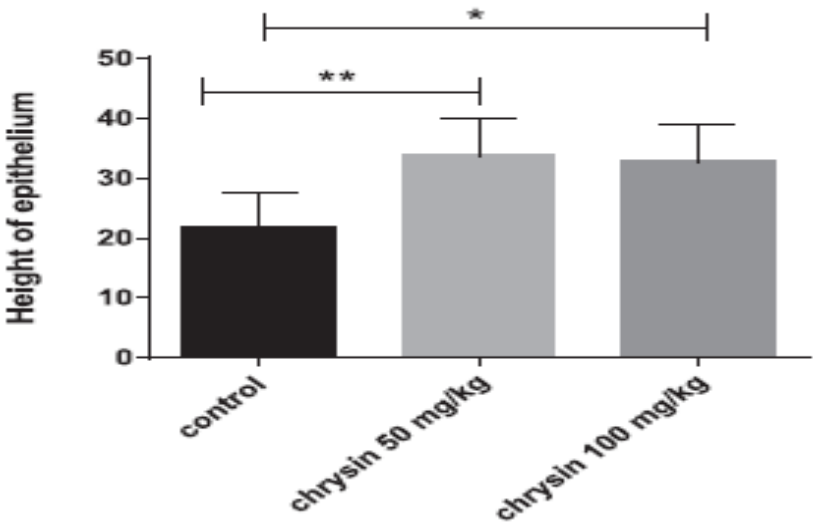

Fig. 5. Height of luminal epithelium (μm) in the uterus of chrysin treated rats compared to vehicle-treated control group rats. * $p \leq 0.05$. ** $p \leq 0.01$

preantral or atretic antral follicles in sections (Fig. 3). Histological examination revealed that the uteri in the control group had wider lumens compared to chrysin-treated groups' uteri despite that rats were sacrificed at the same stage of the cycle (i.e estrous phase) (Fig. 4). Apoptosis was evident in luminal epithelium of 100 mg/kg chrysin-treated group (Fig. 4 F). Also, the height of luminal epithelium was significantly higher in chrysin-treated groups (Fig. 5). Hormone analysis revealed a significant increase in serum concentrations of progesterone in chrysin treated groups (both 100 mg/kg and 50 mg/kg) compared to the control group rats. Similarly, serum concentrations of LH were higher in 100 mg/kg chrysin treated group compared to the control group (Fig. 6). No significant difference was found between groups in estrogen and FSH levels. Immunohistochemical studies showed that no statistically significant difference in the intensity of staining of uterus sections was observed between the control and chrysin-treated groups for both progesterone and ERα receptors (Figs. 7, 8). The myometrium, the endometrium as well as most cells of the luminal epithelium stained positively for progesterone receptor in both control and chrysin-treated animals (Fig. 7 A-C). On the other hand, staining for ERα was observed in the glandular and luminal epithelium and in some cells in the stroma in both control and chrysin-treated rats (Fig. 7 D-F). Similarly, immunohistochemical studies of the aromatase enzyme in ovaries showed that chrysin had no significant effect on its expression (Fig. 8). Positive

staining was intense in follicles, ovarian surface epithelium and corpora lutea in control and

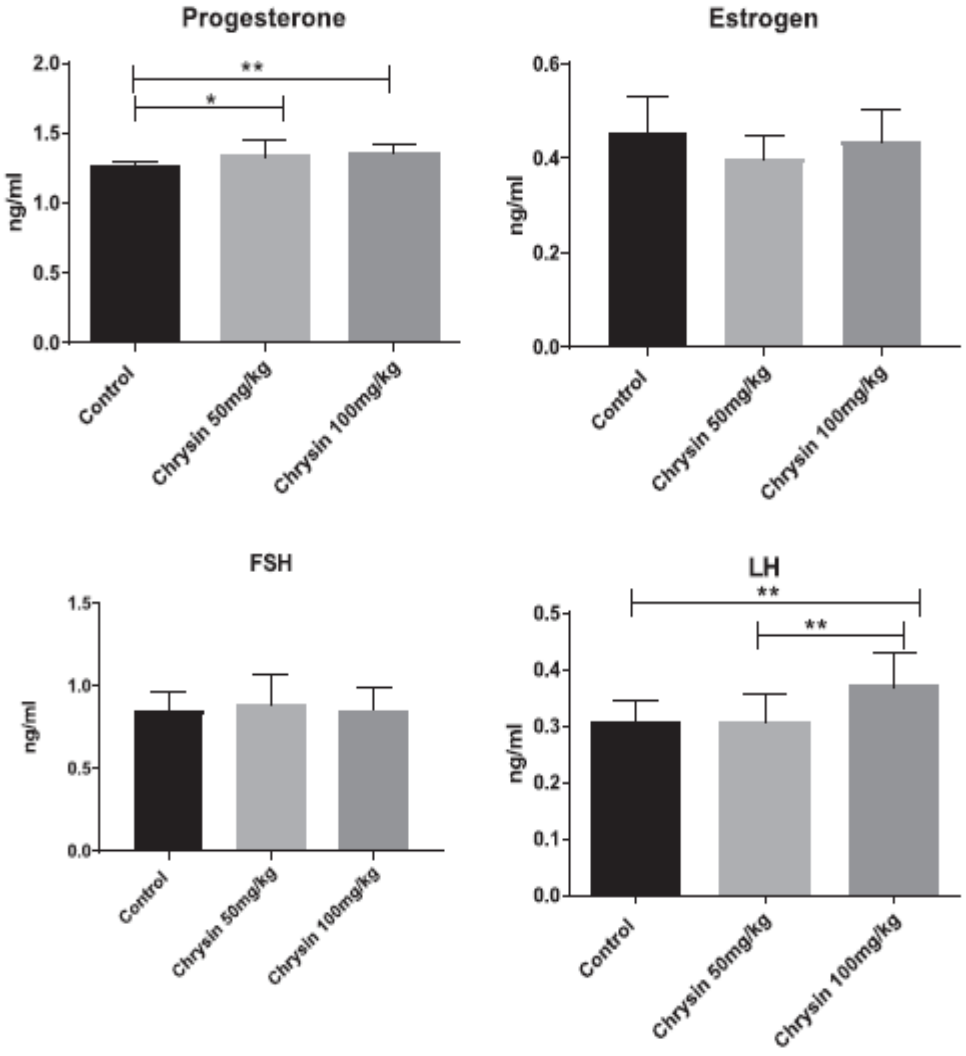

Fig. 6. Effect of chrysin treatment on serum hormone concentrations in female rats in the estrous stage of the cycle. * $p \leq 0.05$. ** $p \leq 0.01$.

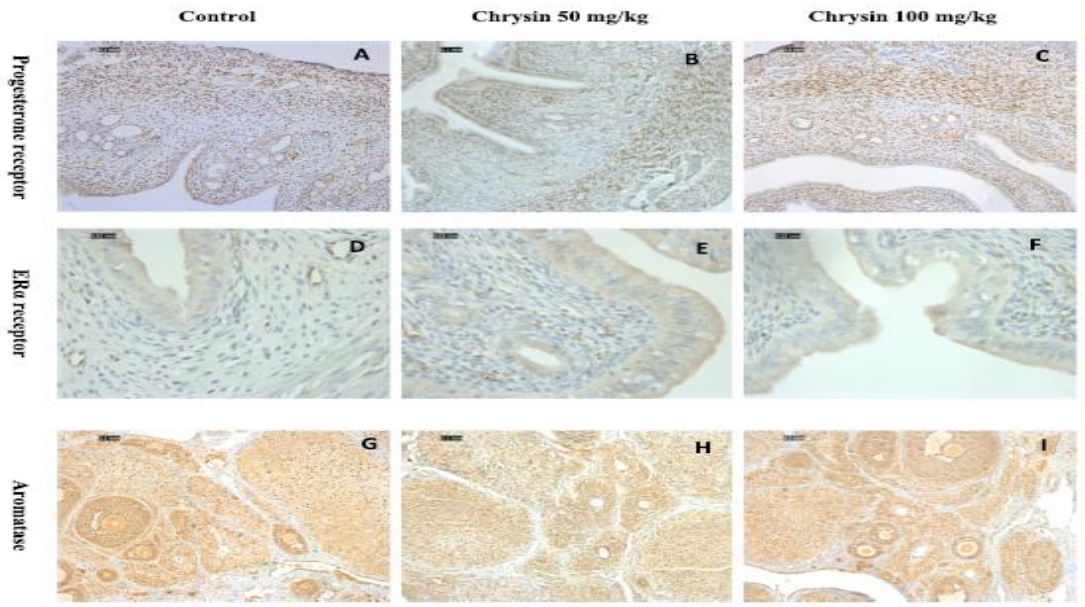

Fig. 7. Representative immunohistochemical images showing the effect of chrysin treatment on progesterone receptor expression in the uterus of female rats (A,B,C), ERα in the uterus (D,E,F) and aromatase expression in the ovary (G,H,I). IHC technique was used in these figures. Scale Barr = 100 μm in figure A,B,C,G,H,I. Scale Barr = 20 μm for D,E,F.

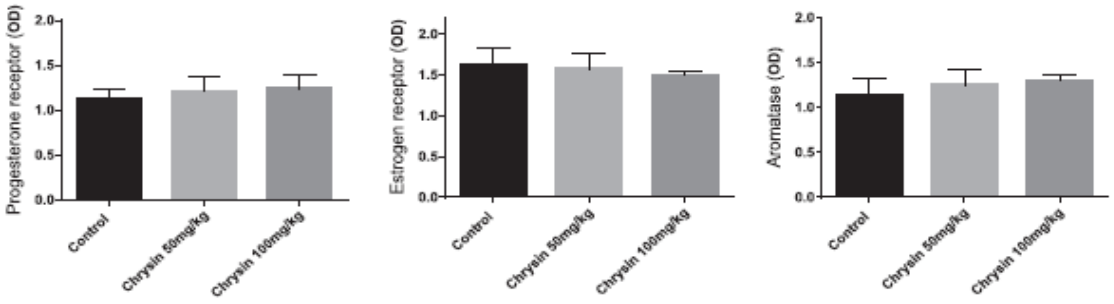

Fig. 8. Results of immunohistochemistry for the determination of relative protein expression. Optical density for progesterone receptor, ERα and aromatase. Data are expressed as mean ± S.D. No significant difference was observed between different groups

chrysin treated groups (Fig. 7 G-I).

DISCUSSION

Estrous cycle is characterized by cyclical changes in the uterus, ovaries, vaginal mucosa, and hormone levels. Vaginal cytology is considered the best standard for staging female animals, as the various estrous cycle stages can be reliably identified from vaginal smears (Singletary et al., 2005). The estrous cycle is divided into four phases: proestrous, estrous, metestrous, and diestrous (Paccola, Resende, Stumpp, Miraglia, & Cipriano, 2018). In this study, the estrous stage lasted for more than 3 days in 60% of rats treated with chrysin (100 mg/ kg) instead of 12 h, the normal duration of estrous phase in rats (Paccola et al., 2018). The change in vaginal smear appearance

reported in this study is most probably due to hormonal changes produced by chrysin. In the present investigation, the uteri of rats in the control group were wide and fluid-filled when animals were sacrificed at the estrous phase of the cycle. On the other hand, chrysin-treated groups had uteri with slit-like lumens. Furthermore, total uterus weight, including its fluid content, was higher in the control group. It is well known that the amount of fluid in rat's uterus increases during the estrous phase (Astwood, 1939) and this agrees with our findings. The lower uterine weight in chrysin-treated groups could be due to the significant increase in progesterone level. Earlier reports provided evidence that fluid secretion in uterus is inhibited by progesterone (Astwood, 1939). It has been reported earlier that chrysin inhibited the activity of aromatase, the enzyme responsible for the conversion of testosterone into estrogen, in human granulosa-luteal cells *in vitro* (Rice, Mason, & Whitehead, 2006). It is not known whether this inhibitory activity of chrysin in females exists *in vivo* or not. Our findings confirm that estrogen level and aromatase expression in the ovary were not affected by chrysin-treatement. Detailed studies on the effect of chrysin on steroidogenesis and metabolism of sex hormones are needed. Hormone-levels in the control group in this study were not significantly different from those reported in other studies for female Wistar rats during the estrous phase of the cycle (Akpantah et al., 2010; Singletary et al., 2005). It is well established that progesterone level relates to histological endometrial maturation (Santoro et al., 2000). The increase in progesterone level in this study correlates with the histologic appearance of uterus. Because corpus luteum formation is dependent on LH for continued progesterone secretion (Behrman, Endo, Aten, & Musicki, 1993), it is expected that high LH level due to chrysin treatment will maintain the corpus luteum for longer duration and this will maintain a high progesterone level. The question now is that if corpus luteum was maintained for longer duration why estrogen levels were not increased? It could be due to the aromatase inhibitory activity of chrysin or its metabolites though it needs further detailed studies to answer this question. In the present study, chrysin had no significant effect on the number of atretic preantral and antral follicles in rat ovary. In fact, ovarian follicle atresia is a hormonally controlled apoptotic process (Hsueh, Billig, & Tsafriri, 1994). This contradicts with the findings that chrysin increased atresia in ovaries of Japanese rice fish by 20%, known as medaka reproductive assay, (Kiparissis, 2001). This contradiction could be due to differences in the regulation of atretic apoptosis in different animal species. Chrysin increased the height of luminal epithelium in our study. This effect could be due to its estrogenic activity reported earlier (Collins- Burow, Burow, Duong, & McLachlan, 2000) and/or to the estrogenic activity of its metabolites. Ultrastructural studies investigation is needed to explore the effect of chrysin on different organelles in luminal epithelium. It is well established that the highest apoptotic index in luminal epithelia occurs during the day of estrus (Mendoza-Rodríguez et al., 2002). Chrysin increased the duration of estrous in the current study. The mechanism by which chrysin increases apoptosis in luminal epithelium of rat uterus is unknown and needs further investigation. The effect of chrysin on *p*53 expression warrants studying since *p*53 plays an essential role in the regulation of apoptosis during the estrous cycle (Mendoza-Rodríguez et al., 2002). The staining pattern and intensity of ERα receptors in uterus during the estrous phase seen in the control and chrysin-treated groups was similar. This agrees with previous studies in which the nuclear staining for ERα was found in both glandular and luminal epithelial

cells as well as in the stroma in which glands are embedded (Pelletier, Labrie, & Labrie, 2000). It has been reported earlier that the immunoreactivity of ERα in luminal epithelium is highest during proestrous and estrous phases of the cycle (Wang, Eriksson, & Sahlin, 2000). Similarly, progesterone receptor staining pattern in the control group and chrysin-treated groups was consistent with the findings of others in which progesterone receptor was localized predominantly in the nuclei of epithelial, stromal, and muscle cells (Ohta, Sato, & Iguchi, 1993).

CONCLUSIONS

The present study suggests that chrysin does not affect serum estrogen concentration or ERα expression in rat uterus. Since chrysin is a dietary component present in large quantities in honey and propolis and sold as a food supplement, there is a need to investigate whether it has hormonal activities in humans or not. The ability of chrysin to increase serum progesterone concentration, as seen in the present study, would be of interest if a similar effect is produced by chrysin in women, especially in the perimenopausal period when progesterone levels are sometimes low. Therefore, future clinical studies are needed to investigate chrysin hormonal effects in pre- and postmenoposal women.

Declaration of Competing Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. *Acknowledgements* This research project was fully sponsored by the Deanship of Scientific Research, Al-Ahliyya Amman University, Jordan (Grant number: 7/ 35/2019-2020). Also we thank Dr. Amanda Sainsbury for critically reviewing the article. *Author contributions* Dr. Manal: Conceptualization, data curation, project administration, writing & funding acquisition, Dr. Khalid: funding acquisition, review & editing, Prof. Ahmad examination of histological sections, Dr. Naghm: examination of histological sections. *Ethical statement* All authors: 1) this material has not been published in whole or in part elsewhere; 2) the manuscript is not currently being considered for publication in another journal; 3) all authors have been personally and actively involved in substantive work leading to the manuscript, and will hold themselves jointly and individually responsible for its content.

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