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Full Length Research Paper

Studies on the protective effects of pollen-originated phytoestrogens on the growth and development of ovary in rats

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This present study was undertaken to investigate the effects of pollen extracts on the development of ovary in the presence of exogenous estradiol benzoate. 45-day old female Wistar rats were administered with estradiol benzoate to induce the pathological status of the ovary. Histological observation of ovarian paraffin sections revealed that interruption of the maturation of follicles was associated with massive follicular atresia. Significantly increased serum FSH level was characteristic of the rats. Exposing to estradiol benzoate also resulted in severe lesion of the liver. Abnormally elevated levels of alanine aminotransferase and aspartate aminotransferase were accompanied by increased liver weight and hepatosomatic index. Extracts of phytoestrogens from pollen exhibited protective effects on ovary and liver tissues. Histological evaluation showed that the inhibition of follicular development was markedly diminished after receiving treatment for 28 days. Serum FSH concentration was restored to the normal level. No significant difference in ovarian weight and ovary-somatic index could be detected between treated groups and the control group. The liver tissue displayed normal morphology and function. Taken together, our results demonstrated pollen-originated phytoestrogens were helpful in re-establishing the hormonal milieu and attenuating the adverse influences of estradiol benzoate on the ovary and liver tissues.

Key words: Estradiol benzoate, phytoestogen, pollen, ovary, liver.

INTRODUCTION

Premature ovarian failure (POF), or premature ovarian dysfunction, caused by wide spectrum of pathogenic mechanisms, such as autoimmune diseases, chromosome and follicle stimulating hormone (FSH) receptor abnormalities, radiotherapy and chemotherapy, are becoming an increasingly concerned global health issue for women (Vegetti et al., 2000; Pal and Santoro, 2002; Chang et al., 2007; Dragojević-Dikić et al., 2010). Estrogen replacement therapy is a common protocol applied to restore the normal hormonal milieu in POF patients (Panay and Kalu, 2009). However, there are concerns that this therapy may increase the risk of breast cancer and venous thromboembolism especially in young women (Bines et al., 1996; Holland, 2001; Charlotte and Margaret, 2002; Appelbaum, 2011). Finding a natural alternative to estrogen replacement therapy has generated substantial interests.

The role of background concentrations of phytoestrogens in the human diet is receiving increasing attention as more and more beneficial effects of phytoestrogens on health have been reported. Diets containing phytoestrogens have been shown to protect

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Abbreviations: EPPs, Extracts of phytoestrogens from pollen; **POF,** premature ovarian failure; **FSH,** follicle stimulating hormone; **E_2,** estradiol; **T,** testosterone; **LH,** luteinizing hormone; **ALT,** alanine aminotransferase; **AST,** aspartate aminotransferase; **CHO,** cholesterol; **GLU,** glucose; **ABW,** average body weight; **OSI,** ovarian-somatic index; **HSI,** hepatosomatic index.

human populations against cancer of the breast and prostate gland (Ingram et al., 1997; Fritz et al., 1998; Wu et al., 2002; Peeters et al., 2003; Limer and Speirs, 2004; Thanos et al., 2006; Cotterchio et al., 2008). Other studies favored their roles in the reduction of endometrial cancer risk (Horn-Ross et al., 2003; Bandera et al., 2009). Their positive effects on preventing bone loss and coronary artery disease were also described (Nakamura et al., 1992; Adlercreutz et al., 1993; Barnes, 1998; Deodato et al., 1999; Arjmandi et al., 2000; Squadrito et al., 2000 and 2002). Pollen from *Pinus yunnanensis faranch*, collected by hands only and rich in phytoestrogens compounds, is a widely accepted traditional healthy food in south-east Asian areas and well known for its nutritional value and therapeutic benefits. Given the growing body of evidence regarding breast cancer and thrombosis risks in the treatment of POF, effects of pollen-originated dietary phytoestrogens on the prevention and treatment of ovarian related problems are worthy to be investigated in depth.

While ideally phytoestrogen effects should be measured by human studies, such studies are often very time consuming and expensive to run. As an alternative, we used laboratory rats as the experimental animal models to study the influences of pollen-originated phytoestrogens on the growth and function of ovary in the presence of exogenous estradiol benzoate.

MATERIALS AND METHODS

Animals

Female Wistar rats of clean grade were purchased from the College of Veterinary Science, Yangzhou University, Jiangsu Province, China (Certificate No. SCXKSU2007-0001). Rats were 45-day old weighing 110 to 122 g. They were raised 3 per cage and maintained at a standard environmental condition (temperature 22 to 25ºC, relative humidity 40 to 60%) with 12 h/12 h dark/light photoperiod. A ventilation system was provided to ensure the airflow in the room. Whole value grain feedstuff and sterilized water were available *ad libitum* over the whole experiment. The use and care of animals followed the “Guide for Care and Use of Laboratory Animal” of the Comparative Medical Centre of Yangzhou University, a registered animal facility for supervising experiments on laboratory animals.

Reagents

Estradiol benzoate (*β*-estradiol-3-benzoate, Sigma chemical company, St. Louis, USA) was first dissolved in isopropanol and then re-suspended in olive oil. Extracts of phytoestrogens from pollen (*P.yunnanensis faranch*) (EPPs) were provided by Yantai New Era Health Industry (Group) Co., Ltd. The concentration of phytoestrogens in 1ml of EPPs was equivalent to that contained in 0.1 g pollen grains with 6% moisture content (patent product, No. 200810022253, mainly prepared as described by Zhou et al. (2010) except that the ratio of solvent to pollen was 10 to 1 instead of 2 to 1. The concentration of estrogen-like compositions present in EPPs at the level of 10^{-9}g/ml).

Solutions were stored at 4°C and adjusted to room temperature just before use.

Administration protocol

A total of 84 rats were assigned randomly into 7 groups of 12 rats each. Every 2 days each rat in the model Group (Gm), Group 1(G1), Group 2(G2) and Group 3(G3), received an intramuscular injection of 200 μg/rat estradiol benzoate in 200 μl of olive oil. This treatment was performed for 6 consecutive times to induce the pathological status of ovarian dysfunction. Besides estradiol benzoate, rats in G1, G2 and G3 also received daily treatment of EPPs at different concentrations. Rats in G1 and G2 received 3 and 2 ml/kg·day EPPs by gavage, respectively. Rats in G3 received 2 ml/kg·day EPPs by intraperitoneal injection. After receiving the last injection of estradiol benzoate on D60, rats in Gm were raised under the standard condition for another 12 days till sacrifice on D73. While rats in EPPs treated groups (G1, G2 and G3) continued their daily EPPs administration till D72. Body weight of each rat was recorded weekly to make adjust of the volume of administration. Three groups of rats were set as vehicle control groups. The same volume of olive oil and saline were administered to rats instead of estradiol benzoate and EPPs, respectively. G0, receiving olive oil by intramuscular injection, was the vehicle control group for Gm. Gg, receiving olive oil by intramuscular injection and saline by gavage was the vehicle control group for G1 (also for G2). Gi receiving olive oil by intramuscular injection and saline by intraperitoneal injection was the vehicle control for G3.

Determination of serum hormone and blood biochemical indicators

On D73, body weight of each rat was obtained in fasting condition. Then all rats were deeply anesthetized with mebumal sodium and sacrificed. Approximately 10 ml of whole blood were obtained from femoral arteries. Serum hormone, estradiol (E_2), testosterone (T), luteinizing hormone (LH) and follicle stimulating hormone (FSH) were detected by chemiluminescence analysis (BC-027_TY6876, Beckman, the United States).

Blood biochemical indicators, alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol (CHO) and glucose (GLU) were determined by auto-blood biochemical analyzer (TBA-120, TOSHIBA, Japan).

Preparation of histological specimen

The tissue samples, including heart, liver, kidney, thymus, bilateral ovaries and uterus were collected and weighed individually. The viscera indexes were calculated using the the following formula:

Viscera Index = Visceral weight (mg)/body weight (g) × 100.

Paraffin sections of liver and ovary were prepared following the standard procedure (Cui et al., 2009). Fixed and paraffin embedded tissues were cut at 5 μm thickness (more than 5 samples per tissue per group) and stained with hematoxylin and eosin (H&E). Histopathological changes were observed under conventional optical microscrope (OLYMPUS BX41, Japan).

Statistical analysis

A standard software package (SAS10.0) was used. All data were presented as mean ± SD.

One-way analysis of variance (ANOVA) was used to pairwise compare differences between different groups. P-values <0.05 were considered significant.

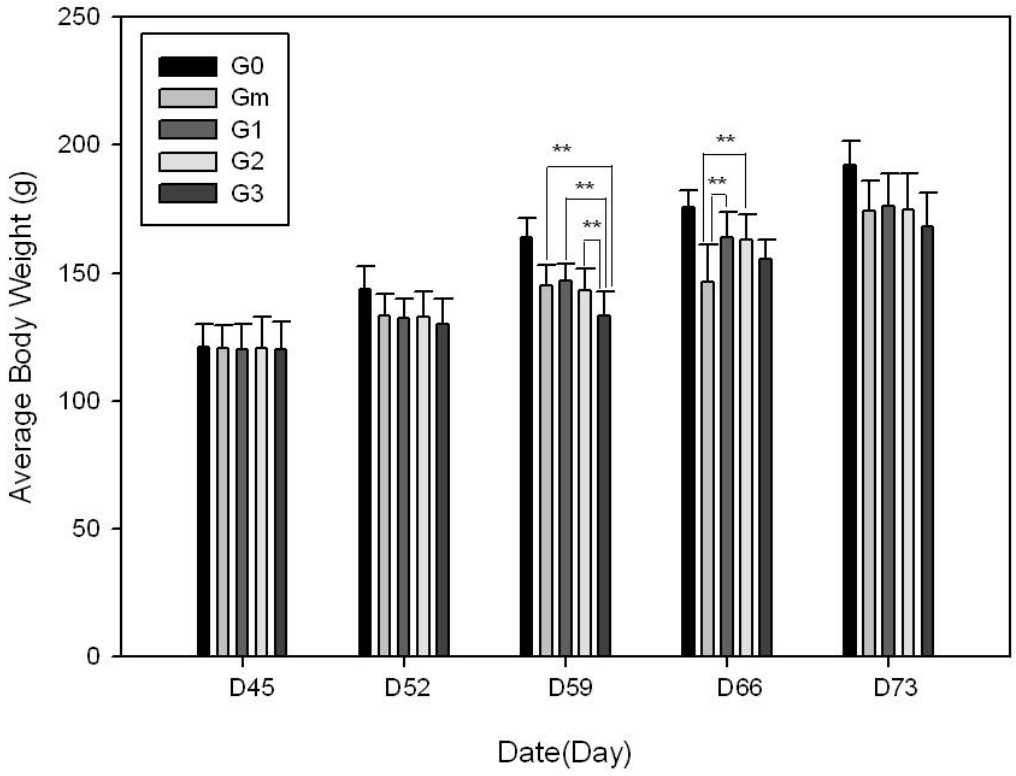

Figure 1. Changes in the rat body weights during 28days of experiment. Data are expressed as mean ± SD (n=12). ** P<0.01, as compared between two groups indicated.

RESULTS

Changes of body weights during 4 weeks of experiment

At the beginning of the study, there was no significant difference in the average body weight (ABW) between different groups. Weekly body weight monitoring showed that rats in the vehicle control groups always had significantly higher ABW than rats in the corresponding estradiol benzoate or EPPs treated group(s) (P<0.05). No significant difference in ABW could be detected among three vehicle control groups. This was shown clearly in Figure 1 by the apparently much higher bars representing the G0 (bars representing Gg and Gi were omitted for the concise of the figure). We assumed that stress caused by daily treatments like gavage and intraperitoneal injection was not the main reason which resulted in the loss of body weight.

On D59, no significant difference in ABW could be detected between Gm, G1 and G2. However, rats in G3 had the lowest ABW (shown in Figure 1). Since the only difference between G2 and G3 was the way of EPPs administration, we concluded that rats responded a bit more strongly to the intraperitoneal injection than to the gavage treatment during the earlier experimental stage. As rats became gradually acclimatized to the treatment, compensations for growth were made thereafter. On D66, no significant difference in ABW could be observed within three EPPs treated groups and differences in body weight were eventually eliminated at the end of the experiment.

An excessive amount of estradiol benzoate was administered to rats in order to induce the pathological status of ovarian failure. We found that this exogenous hormone had a negative effect on the growth of rats. On D66, rats in Gm had stopped receiving estradiol benzoate for 6 days. However, rats in this group still had the lowest ABW (146.6±14.52 g) and remained almost the same as that on D59 (145.20±8.00 g).

Effects of different treatments on viscera indexes

Three vehicle control groups (G0, Gg and Gi) had no significant difference in the average weights of ovary/liver and their somatic indexes. Only data in G0 were used to show the results (Figure 2A and 2B).

The average weight of bilateral ovaries in Gm was 0.06±0.03 g which was significantly lower than that in G1 (0.10±0.03 g) and G3 (0.10±0.02 g). After treated with EPPs, rats in G1 and G3 obtained almost the same normal ovarian weight as rats in G0 (0.12±0.03 g). The same profiles of differences could be observed in the OSI results (Figure 2A). Our results indicated that EPPs

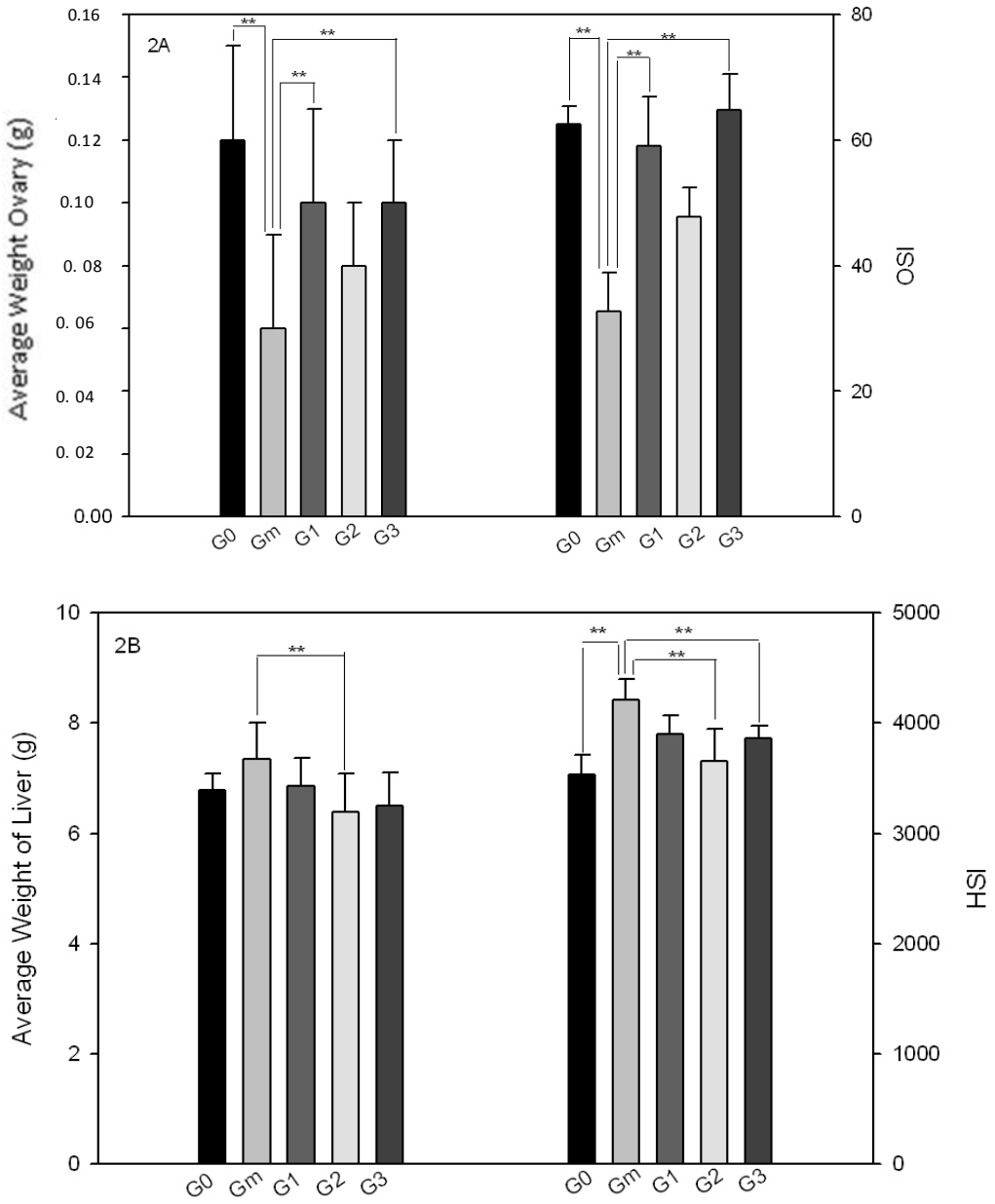

Figure 2. Comparisons of average weights and somatic indexes of ovary and liver between different groups. (2A), ovary; (2B), liver. Data are expressed as mean ± SD (n = 12). ** P<0.01, as compared between two groups indicated. OSI: ovarian-somatic index. HSI: hepatosomatic index.

could counteract the adverse effect of estradiol benzoate on the development of ovaries. Since rats in G2 (receiving less daily amount of EPPs than rats in G1) did not increase the average ovarian weight to a statistically significant level (0.08±0.02 g), we inferred that the effect of EPPs was dose-dependent.

While no significant difference in the liver weight and HSI (hepatosomatic index) could be detected among G0 and EPPs treated groups, abnormally higher values of average liver weight (7.34±0.66 g) and HSI (4212.64±183.94) were observed in rats from Gm (Figure 2B). We found that exogenous estradiol benzoate exerted a negative impact on the development of liver. After intervened by EPPs, liver weight and HSI were capable of restoring to the normal levels.

Rats from different groups showed no significant difference in the average weights of heart, kidney and uterus (data not shown). As for the thymus, rats in three vehicle control groups had significantly higher organ weights and thymus-somatic indexes (P<0.05) than that in Gm and EPPs treated groups. Our results indicated that estradiol benzoate could influence the development

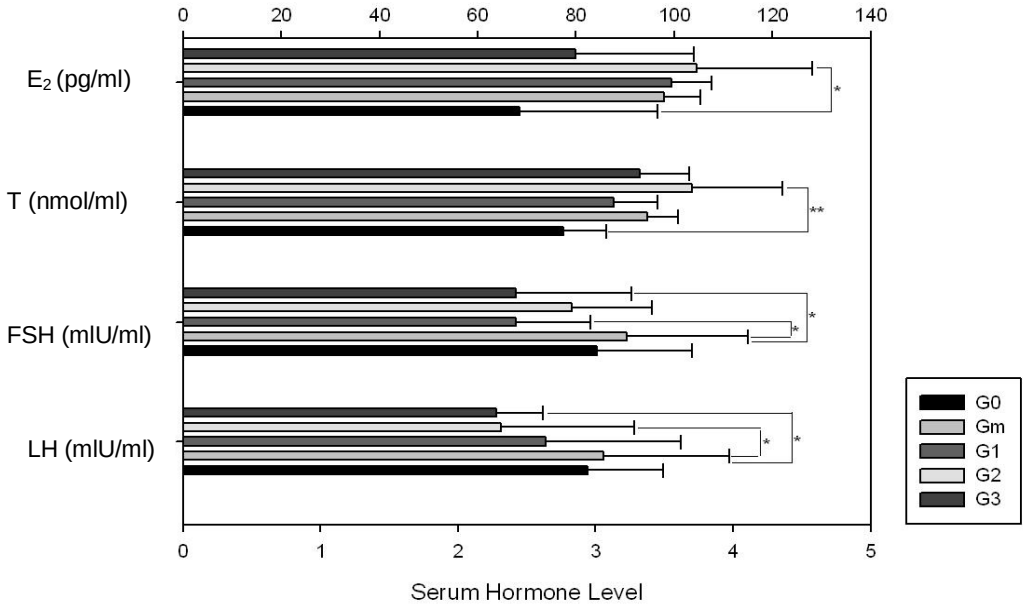

Figure 3. Comparisons of serum hormone levels between different groups. Data are expressed as mean ± SD (n=12). ** P<0.01and * P<0.05, as compared between two groups indicated.

Table 1. Concentrations of serum biochemical indexes in different groups.

Group	G0	Gm	G1	G2	G3
ALT(U/L)	36.30±4.00	41.9±10.12	35.82±8.58	29.73±3.80	24.56±2.00
AST(U/L)	96.00±21.08	113.5±36.28	82.82±19.74	106.36±26.38	92.78±9.58
CHO(mmol/L)	1.73±0.10	2.06±0.27	1.86±0.18	1.75±0.19	1.55±0.12
GLU(mmol/L)	7.14±0.61	7.09±0.82	6.65±0.49	6.31±0.68	6.78±0.59

of this organ indirectly and no protective effect of EPPs was observed.

Comparisons of serum hormone levels

Rats from three vehicle control groups had almost the same serum hormone levels. Data from G0 were used to plot the chart (Figure 3).

As an indicator of ovarian dysfunction, a significantly elevated FSH level was detected in rats from Gm (3.23±0.88mIU/ml) associated with an increased LH concentration of 3.06±0.91 mIU/ml (Figure 3). Compared with G0, EPPs treated groups showed no significant difference in serum FSH and LH concentrations. We attributed the establishment of normal serum hormone levels to the impact of EPPs. A dose-related effect of EPPs was observed. The FSH level in G2 was lowed to 2.83±0.58 mIU/ml due to the treatment of EPPs. However, it was not significantly reduced as that in G1 (2.42±0.54 mIU/ml). Similarly, when E_2 and T levels were compared, no significant difference could be detected among rats from different groups except that rats in G2 remained significantly higher E_2 and T concentrations than that in G0. These results further demonstrated that EPPs were helpful in restoring the normal hormonal milieu.

Comparisons of serum biochemical indicators

Serum biological indicators were determined mainly for the monitoring of liver function. Rats from three vehicle control groups showed almost the same regularity of variation. Data from Gg and Gi were not shown in Table 1. Significantly higher levels of ALT and AST were observed in rats from Gm. Briefly, ALT concentration in Gm was significantly higher than that in G2 and G3 (P<0.01) and AST concentration higher than that in G1 (P<0.01). Particularly concentrated in liver, ALT and AST are two enzymes commonly used as important markers for liver health. Their elevated levels specifically reflected the abnormal status of liver.

Compared with G0, G2 and G3, a significantly higher CHO level was founded in Gm (P<0.01). This was consistent with the abnormally high liver weight and HSI

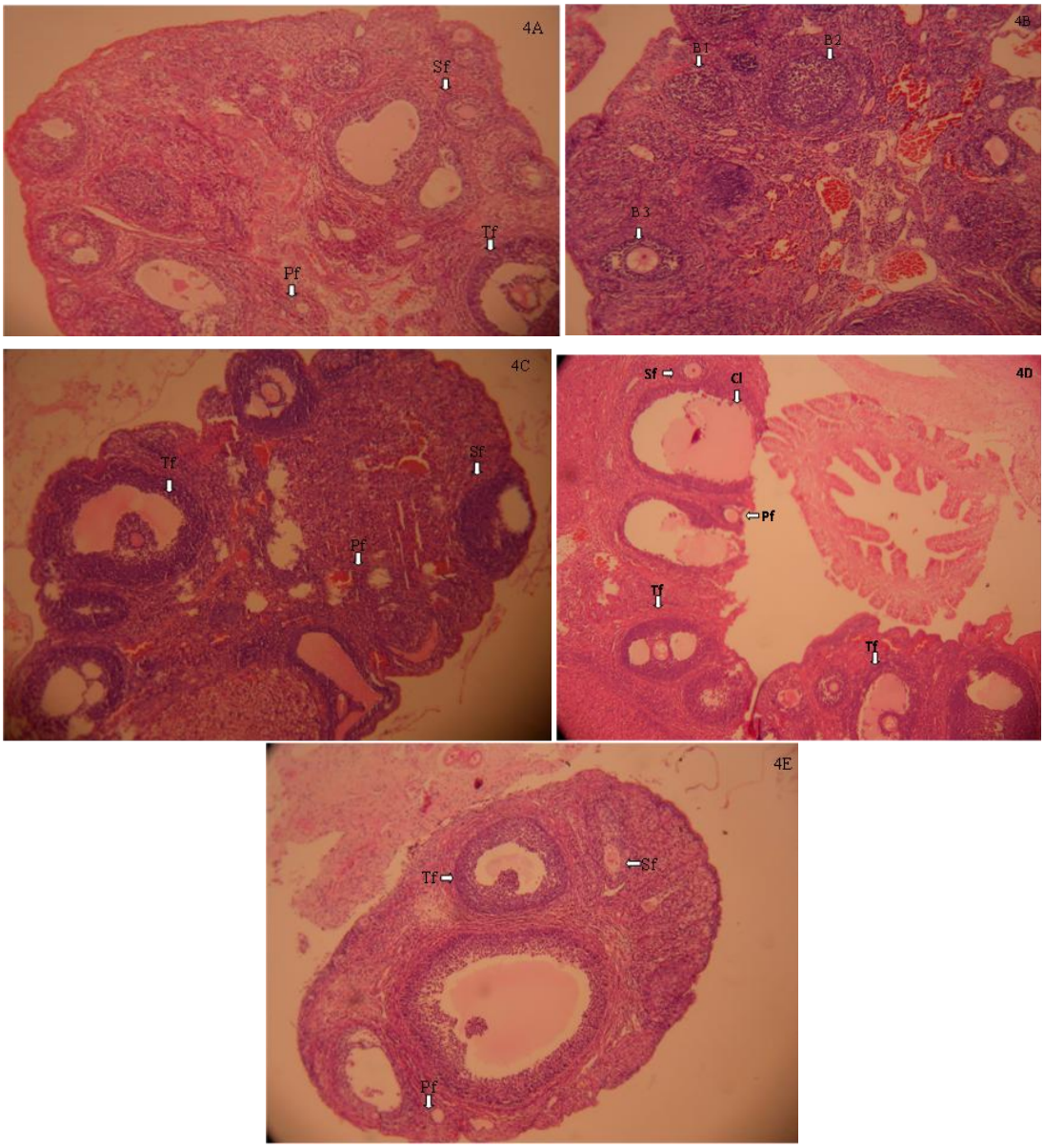

Figure 4. Histopathological sections of rat ovary from G0, Gm and EPPs treated groups. (4A-4E), ×100. Histological sections from more than 5 rats per group were checked. (4A) G0; (4B) Gm; (4C) G1; (4D) G2; (4E) G3. Pf: primary follicle; Sf: secondary follicle; Tf: tertiary follicles; Cl: corpus luteum. A massive follicular atresia was observed in Gm: irregularly shaped follicles (B1), atrophy of granulosa cells (B2), shrunken and dark nuclei (B3).

of this group, which reflected the pathological status of the liver. Lower levels of GLU were found in three EPPs treated groups. We concluded that EPPs had positive effects on liver function especially in the metabolism and regulation of blood cholesterol and glucose.

Histopathological observations of liver and ovary

Rats from G0, Gg and Gi had normal tissue morphologies. We used histological paraffin sections from G0 to explain the results.

Follicles at different developmental stages could be found in ovarian sections from G0 (Figure 4A). Rats from Gm had relatively small-sized ovaries associated with degenerate ovarian development (Figure 4B). Primordial follicles could be detected, while primary and secondary follicles were sparse. A massive follicular atresia was observed: irregularly shaped follicles, atrophy of granulose cells, and pyknotic nuclei (shrunken and dark).

In ovarian sections from EPPs treated groups, primordial follicles formed the majority of the follicles. Follicles at different phases of development could be detected (Figure 4C, D and E). There were primary follicles surrounded by layers of prismatic epitheliums, secondary follicles with multiple layers of granulosa cells and tertiary follicles with visible fluid-filled cavity (the antral follicle) and cumulus oophorus. Histological observations validated our former conclusion about the positive effect of EPPs on the development of the ovary.

At necropsy, the liver tissues from G0 were soft with normal pinkish-brown color and even texture, whereas liver tissues from Gm exhibited tumefaction and light color. Sporadic yellow stains on the hepatic surface which resembled the symptom of fatty liver were characteristic of livers from Gm. No distinguishable optical symptoms could be found in liver tissues from three EPPs treated groups except that they were slightly smaller in size than that in the vehicle control groups.

Microscope observations of paraffin sections revealed that normal hepatic sinusoid and hepatic lobules could be found clearly in liver tissues from G0 (Figure 5A). Abnormalities related with fatty degeneration were universally observed in livers from Gm, such as formation of hepatocellular hypertrophy, congestion in the hepatic sinusoid and cytoplasmic vacuolation. The nucleus was pushed to one side of the hepatic cell by droplet of fat and the normal polygonal hepatic cell changed its morphology (Figure 5B). In EPPs treated groups, hepatic cords were visible again. The accumulation of fat in hepatic cells was reduced or disappeared (Figure 5C, 5D and 5E). Compared with G1 and G2, liver tissues from G3 displayed the optimum state.

DISCUSSION

During the entire experimental period, no abnormal activities or significant difference in ingestion, excretion or psychosis were observed in rats from different groups. Our results showed that rats in Gm (administered with estradiol benzoate and received no treatment of EPPs) suffered impaired ovarian growth and development. Twelve days after removal from the exogenous estrogen, the pathological signs were still not resolved. Significantly decreased ovarian size and ovary-somatic index were accompanied by abnormalities in histological appearance. On the contrary, rats in three EPPs treated groups (exposed to estradiol benzoate for the same period) were recovered and obtained the normal ovarian morphology and resumption of ovarian activity. We concluded that EPPs could provide protection against ovarian dysfunction caused by imbalanced estrogen level.

EPPs used in our study contained sufficient phytoestrogens derived from pollen (*P. yunnanensis faranch*). Phytoestrogens are capable of showing both estrogenic and anti-estrogenic effects. The balance between the two activities is determined by the ratio of phytoestrogen to estrogen. They may exert antiestrogenic effects in high-estrogen environments and have weak estrogenic effects in low-estrogen environments (Messina et al., 1994). Since rats in G1, G2 and G3 were administered with estradiol benzoate they had relatively high circulating concentrations of estradiol. It may be expected that anti-estrogenic effects would dominate in rats from these groups. This explained why rats from EPPs treated groups were saved from the substantial deleterious effects caused by excessive amount of estrdiol benzoate on the ovary. The anti-estrogenic role of phytoestrogens was in conformity with previous research in rats (Sharma et al., 1992).

Rats in G2 and G3 received the same daily amount of EPPs by gavage and intraperitoneal injection, respectively. In our study, difference in the efficacy of administration was observed with intraperitoneal injection of EPPs showing better effects on the development of ovary than did the gavage treatment. G3 had not only relatively higher average ovarian weight, but also higher ovarian-somatic index than G2 (64.77±15.73 and 47.83±14.58, respectively). When body weight was considered, rats in G3 even had the largest weight gain of 34.67 g for the last two weeks compared to rats in G0(28.53 g), Gm(28.94 g), G1(29.22 g) and G2 (31.61 g), respectively. As to the serum hormone concentrations, rats in G3 always exhibited relatively lower values than rats in G2. The decreased FSH level of 2.42±0.84 mIU/ml of G3 was equaled to the level of G1 (2.42±0.54 mIU/ml). This indicated that pharmacologically relevant concentrations may not be fully achieved following oral administration which reduced the bioavailability of phytoestrogens unless increasing dose amount. Consistently, we noted that dose of consumption was of importance for the protective effects attributed to EPPs. This dose-response was also reported in another study in which higher phytoestrogen intakes were associated with greater reductions in breast cancer risk (Thanos et al., 2006). We concluded tissue absorption of intra peritoneally administered EPPs could provide more efficient effects on ovarian growth and development.

Studies had shown that phytoestrogens were involved in cell-mediated inflammatory autoimmune disease and exerted inhibitive effects in atherosclerosis, arthritis, and Alzheimer's disease (Breinholt et al., 2000; Belcher and Zsarnovszky, 2001; Bhathena and Velasquez, 2002; Howes et al., 2003; Muthian and Bright, 2004). We had expected a positive effect of EPPs on the growth and development of thymus. In our study estradiol benzoate did inhibit the growth of thymus (shown by significantly lower weight of this organ in Gm and three EPPs treated groups than the control groups). However, no protective effect was detected and there was no significant difference in the thymus weight or thymus-somatic index between EPPs treated groups and Gm. Further in-depth studies are needed to understand it better.

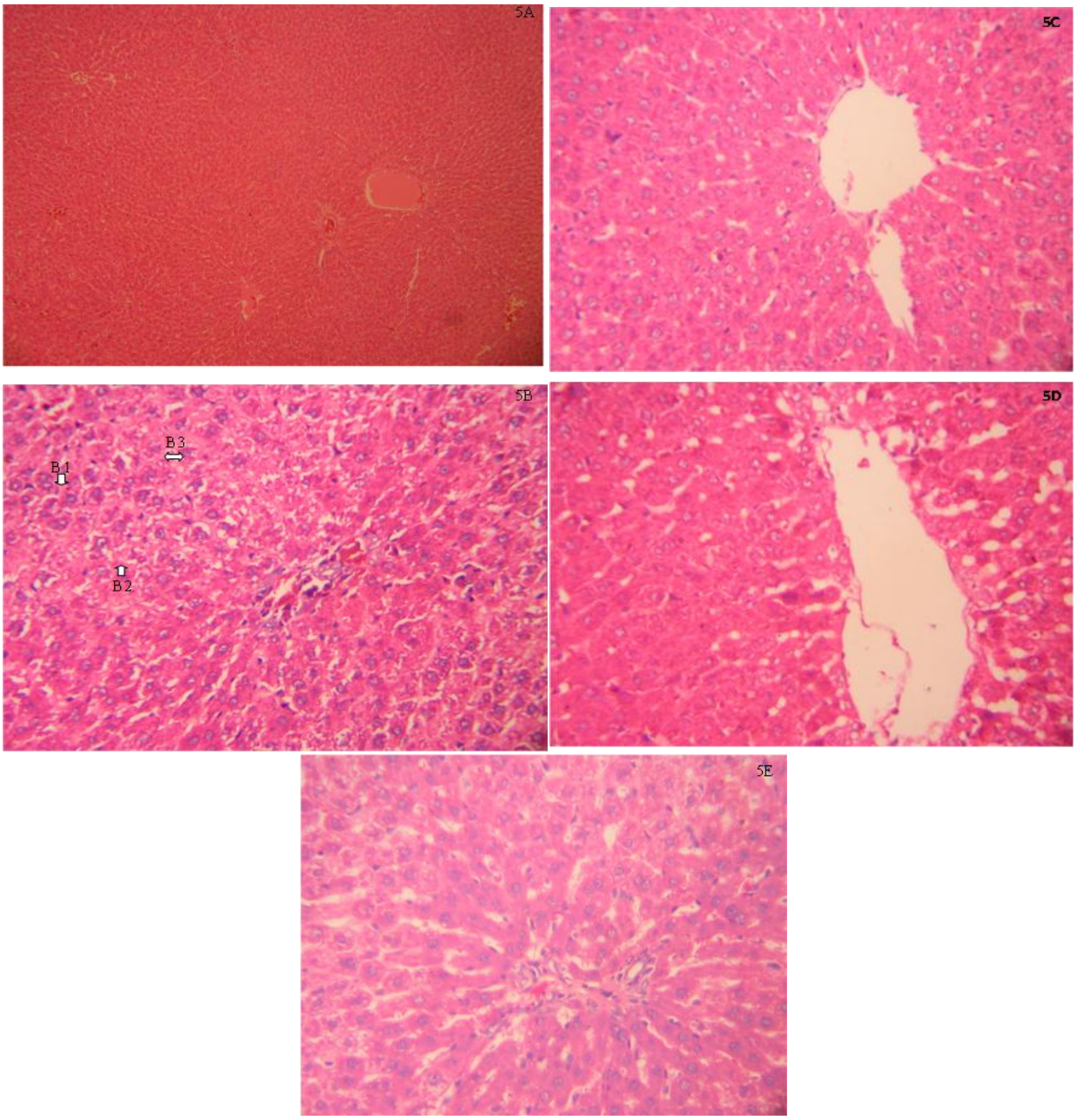

Figure 5. Histopathological sections of rat liver from G0, Gm and EPPs treated groups. (5A), ×40; (5B-5E), ×100. Histological sections from more than 5 rats per group were checked. (5A) G0; (5B) Gm; (5C) G1; (5D) G2; (5E) G3. Compared to normal hepatic sinusoid and clear hepatic lobules in G0 (5A), histopathological changes due to fatty degeneration were observed in Gm, mainly including hepatocytic hypertrophy (B1) congestion or disappear of hepatic sinusoid (B2), cytoplasmic vacuolation (B3).

Up till now, a number of biologic mechanisms possibly involved in the reduction of human breast cancer by phytoestrogens intake have been postulated, such as the decrease of tissue sensitivity to environmental stimuli, the antioxidant properties of phytoestrogen and the suppression of functional genes related to tumor progression (Murrill et al., 1996; Lamartiniere et al., 1998; Hilakivi-Clarke et al., 1999; Messina and Loprinzi, 2001). Our study showed that ovary and liver were targeted organs for estrogen action. Phytoestrogens from pollen were helpful in re-establishing their normal functions in the presence of exogenous extrogen. However, there is a paucity of interaction studies in this area. Little is known about the interaction between metabolisms of functional compounds in phytoestrogens and factors that can affect the response of the animal. This partly explained why

effects of phytoestrogens remained controversial in some cases. We suggest further investigation focus on individual chemical assay and therefore can offer a valuable guide to the estrogenicity of EPPs compounds. It is also demanding to build up an analytic pollen composite database to make adequate dietary phytoestrogen assessment possible.

Conclusion

Results from the present investigation in an *in vivo* rodent model supported the therapeutic role of phytoestrogens from pollen in obtaining normal ovarian and liver functions in the presence of exogenous estrogen. We suggested dietary phytoestrogens intake in the form of pine pollen may reduce the risk of developing estrogen related hormone problems. The consumption of pollen products may be applied to POF patients. Data presented in this paper was useful for the design of future dietary and clinical trials of pollen for more safety and efficacy.

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