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The ameliorative effects of bee bread supplement on the ovarian dysfunction induced by high-fat diet: Comparison with *S. officinalis* and shifting to normal diet supplement

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ABSTRACT

Bee bread, a naturally fermented bee product, could alleviate obesity-related disorders but its protective role against high-fat diet (HFD)-induced ovarian dysfunction has not been studied yet. Thirty-eight Wister albino female rats were divided into control and HFD groups. The control group (n=7) received a normal diet for 14 weeks whereas HFD group was supplemented with 40% HFD for 10 weeks and then subdivided into 4 subgroups that received either HFD or HFD and *S. officinalis* (300 mg/kg) or HFD and bee bread (0.5 gm/kg) or normal diet for another 4 weeks. Histopathological examination of ovarian tissues was done. The estrus cycle, final body weight, lipid profile, fasting serum glucose, and reproductive hormone levels were investigated. mRNA expression of TNF-α and IL-6 in the blood and the ovarian cytochrome P450 family 17 subfamily A member 1 (CYP17A1), cyclin D1(CCND1) and autophagy-related protein-5 (Atg5) were determined. Immunohistochemical assessment of nuclear factor-kappa B (NF-κB), peroxisome proliferator-activated receptors gamma (PPARγ), and caspase-3 was done in all groups. Irregular estrous cycle altered folliculogenesis and reproductive hormones, dyslipidemia, and increased body weight were detected in HFD group. Also, upregulation of NF-κB, TNF-α, IL-6 and caspase -3 expressions, and downregulation of CCDN1, Atg5 and PPARγ were observed in HFD group. Bee bread, *S. officinalis* and to a lesser extent normal diet supplements bee bread improved ovarian dysfunction induced by HFD by inducing autophagy and steroidogenic genes and inhibiting inflammation and apoptosis. Bee bread and *S. officinalis* could be protective agents against ovarian dysfunction and obesity induced by HFD.

Introduction

Dietary practices have detrimental impacts on fertility (Silvestris *et al*., 2019). Males and females' fertility can be deteriorated by a diet high in fat, independently of obesity (Skaznik-Wikiel *et al*., 2016).

HFD consumption has been linked to increased inflammation (Hohos *et al*., 2020). Both low-grade systemic inflammation (Skaznik-Wikiel *et al*., 2016) and inflammatory cell infiltration in primordial follicles, deteriorate ovarian quality (Snider and Wood, 2019). Local adipose tissue macrophage cells secrete cytokines as tumour necrosis alpha (TNF-α) and induce nuclear factor-kappa B (NF-κB) (Tomasello *et al*., 2023). On the hormonal level, impairment of the hypothalamic-pituitary-ovarian (HPO) axis affects folliculogenesis, leading to a depletion in the primordial follicle pool following HFD consumption (Hohos and Skaznik-Wikiel, 2017). Furthermore, obesity may disturb the microvasculature (Velazquez *et al*., 2023) and cause mitochondrial dysfunction (Elías-López *et al*., 2023), resulting in oocyte dysfunction.

Nuclear factor-kappa B (NF-κB) is a transcription factor, engaged in metabolism and inflammation. Many studies have recorded that HFD is a stimulator for NF-κB in adipose tissue (Tomasello *et al*., 2023) and reproductive organs (Gao *et al*., 2021). While Peroxisome proliferator-activated receptors (PPARs) act as ligands activate transcription factors and regulate metabolic processes (Vitti *et al*., 2016). PPARγ is known as a regulator of cellular functions including adipogenesis (Yoon *et al*., 2020). In the ovary, PPARγ is highly expressed in granulosa cells while lower expression is in theca cells and corpus luteum in the ovary of rodents and ruminants (Komar *et al*., 2001; Froment *et al*., 2003; Froment *et al*., 2006). PPARγ has a main role in linking lipid metabolism and reproduction (Froment *et al*., 2006). HFD alters the function of PPARγ and gene expression (Hohos and Skaznik-Wikiel, 2017).

Numerous genes can be impacted by a high-fat diet. For instance, the cell cycle gene, autophagy gene, and steroidogenic gene. Altered expression of the genes important in steroidogenesis, such as the cytochrome P450 family 17 subfamily A member 1 (CYP17A1) gene, which encodes a key enzyme in the synthesis of steroid hormones (Asemota *et al*., 2020) and relates to visceral and subcutaneous fat accumulation, was previously reported as a reason for HFD-related infertility (Wang *et al*., 2018). Additionally, HFD-induced obesity resulted in cell cycle arrest. The Cyclin D1 gene (CCND1), which is necessary for progression through the G1 phase of the cell cycle, is altered by HFD, leading to impaired ovarian function (Wu *et al*., 2015). Autophagy has been functionally connected to adipogenesis and obesity; hence, its ablation exacerbated the inflammatory activation and mitochondrial dysfunction caused by HFD (Yamamoto *et al*., 2017). Diet-induced obesity in mice leads to genetic disruption of autophagy genes such as Atg5 and Atg7 (Zhang *et al*., 2013).

Bee bread is a fermented bee product that is produced from plant pollen, honey, and bee saliva (Urcan *et al*., 2018). It also contains phenolic compounds and has been shown to offer anticancer, antimicrobial, and hepatoprotective effects (Othman *et al*., 2019). Bee bread demonstrates anti-ageing activity, contains antioxidants, regenerates all cells of the body (Kieliszek *et al*., 2018), and significantly reduces the obesity index and levels of the lipid profile (Othman *et al*., 2019). *Salvia officinalis* L. is one of the herbs that have been traditionally used to treat and improve infertility issues (Al-bediry and Al-Maamori, 2013), as it has estrogenic activity (Sabry *et al*., 2022) and can affect folliculogenesis and steroidogenesis processes (Alrezaki *et al*., 2021).

To date, minimal research has been done to determine the beneficial effect of bee bread supplementation on the reproductive system and estrous cyclicity. Based on the previously mentioned data, our research project was designed to study the effect of HFD on ovarian function, gene expression, reproductive hormones, and the estrous cycle, and to explore the role of bee bread, *Salvia officinalis*, and caloric restriction in mitigating HFD-induced ovarian dysfunction.

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Materials and methods

Animals and diets

Thirty-eight female Wistar albino rats (4 weeks old) weighing about 80-85 g were obtained from The Animal House of the Faculty of Veterinary Medicine, Assiut University, Egypt. The rats were maintained in an environment with a 12/12 light/dark cycle at 21.0±2.0 room temperature and 60.0±5.0 humidity. All animal experiments followed the ARRIVE guidelines and were carried out by the National Research Council's Guide for the Care and Use of Laboratory Animals. Care and management of the animals were provided according to the guidelines of the Animal House of Assiut University. The study was approved by the Ethical Committee of the Faculty of Medicine at Assiut University. The ethical approval number was 17101769.

After 2 weeks of adaptation period, rats were randomly divided into 2 groups.:

The control group (n.= 7) received the basal diet, which contained 21% protein, 4.6% fat, and 3.45% carbohydrates (2.950 kcal/gram ad libitum) for 14 weeks.

The HFD (HFD-fed group) (n.=31) that was supplemented with HFD for 14 weeks. The HFD was composed of a 55% basal diet, 25% beef tallow, 5% roasted peanuts, 5% milk powder, 5% egg, 3% sesame oil, and 2% NaCl (Ragab *et al*., 2015). One kilogramme of HFD was prepared as follows: 250 g of beef tallow was melted by heating until it became liquid, then it was saturated with 550 g of basal diet, 30 g of sesame oil, 50 g of eggs, and 20 g of NaCl salt. In the end, it was coated with the addition of 50 g of milk powder and 50 g of roasted peanuts. supplementary graphical abstract.

After 10 weeks of HFD feeding, 3 rats were randomly selected and sacrificed at the diestrus stage. The HFD-fed rats were divided into four subgroups and received different supplements until the end of the 14th week from the beginning of the study; the HFD group supplemented HFD (Hohos *et al*., 2018). The HFD and *S. officinalis* extract-treated group received HFD with *S. officinalis* extract (300 mg/kg b.w.t. as a single dose/day) via oral gavage (Salah *et al*., 2016). The HFD and bee bread-treated group received HFD with bee bread in a dose of 0.5 g/kg b.w.t./day via oral gavage (Othman *et al*., 2019), whereas the HFD/ND group shifted from the high-fat diet (HFD) to the normal diet (ND). The study continued for 14 weeks, by the end of the study, animals were fasted for 12 h. then sacrificed when they were at the diestrus stage.

Preparation of Salvia officinalis extract

Salvia officinalis was purchased from the local herbal market, then the leaves of *Salvia officinalis* L were air-dried and extracted by adding boiling water to dried leaves, and then left to cool. Extract water was given by oral gavage. The dose was 300 mg/kg b.wt. and each rat received 1 ml which contained about 55.22 mg (Alzergy *et al*., 2019).

Preparation of bee bread

The Bee bread was purchased from a local stingless bee farm, and samples were collected from honeybee colonies of the local stingless bee farm located in Assiut governorate, Egypt. It was collected during the period from June to October. It was collected manually from combs (Elsayed *et al*., 2021). It was air-dried and preserved at room temperature until the time of use (Smati, 2022). Bee bread was crushed and mixed with distilled water to be suitable for administration by gavage according to the designed dose, 0.5gm/kg b.w.t. Its concentration was 0.092 gm/ml for each rat (Capcarova *et al*., 2020).

Assessment of the estrous cycle

The assessment of the estrous cycle phases was performed for 10 successive days twice during the current study. The first assessment was done at the 9th and 10th weeks after HFD feeding to detect the ovarian dysfunction that induced by HFD feeding. The second assessment was after the third week of treatment with *Salvia officinalis*, bee bread, or restriction to a normal diet (The 13th,14th week). The assessment was performed between 8:00 and 9:00 am daily using both visual assessment and vaginal cytology. The visual assessment determined the degree of vaginal swelling, the color and moistness of the tissues, the size of the vaginal opening, and the presence or absence of visible cellular debris in the vagina, which are all factors that are considered in identifying the stage of estrous (Ajayi and Akhigbe, 2020). Vaginal cytology was done by use of 0.2 ml of saline which was drawn into the pipette, the tip of the pipette was gently inserted into the vaginal orifice at a depth of approximately 5-10 mm, and then the saline is flushed into the vagina and back out 2–3 times. One drop from lavage for each rat was collected and placed on a slide (supplementary Fig. 2). The slides were stained with Giemsa blood stain according to Nelson *et al*. (1982). The estimation of the phase of the estrous cycle is based on the proportion of the cell type present in the vaginal secretion. Rats were determined to be in proestrus when nucleated cells were the predominant cell type, in estrus when cornified cells were predominant, in metestrus when cornified cells and leukocytes were predominant, and in diestrus when leukocytes were predominant (Auta and Hassan, 2016).

Assessment of body and ovarian weight

Rats were weighed every week throughout the time of the experiment. Then, at the end of the 14th week, rats were sacrificed when they were in the diestrus stage, and the ovaries were removed, stripped of fatty tissue, and weighed.

Blood collection

The fasting blood samples (2.5-3ml) were drawn from the rat's retro-orbital sinus using a capillary tube and divided into 2 parts. One part (1ml) of the blood was collected on tubes containing anticoagulant (EDTA) and stored at -80°C for RNA extraction. The other part was collected on plain tubes for serum isolation, and the tubes were centrifuged at 3000 rpm for 15 minutes. The serum was stored at -20°C until the estimation of the fasting blood glucose, lipid profile, and reproductive hormones.

Tissue Collection

The rats were sacrificed in the diestrus stage, and one ovary was collected in Eppendorf tubes which contained RNAlater® Solution, which was kept at 4°C overnight and then stored at -80°C for RT-qPCR analysis. On the other hand, the other ovary was collected in 10% neutral buffered formalin and then processed for histopathological and immunohistochemical examinations.

Biochemical analysis

Serum hormone assays

To assess changes in the levels of estradiol (E2), luteinizing hormone (LH), follicle-stimulating hormone (FSH) and progesterone in the serum, rodent ELISA kits (CUSABIO (China), Catalogue Number CSB-E05110r, CSB-E12654r, CSB-E06869r, and CSB-E07282r, were used respectively following manufacturer's protocols. The sensitivity, inter-assay and intra-assay coefficient of variation (CV) for serum assays are sensitivity is less than 40 pg/ml, less than 0.15 MIU/ml, less than 0.039 ng/ml and less than 0.2 ng/ml. The inter-assay coefficient of variation (CV) are < 15%, < 15%, < 8% and < 15% and the intra-assay coefficient of variation (CV) are < 15%, < 15%, < 10% and < 15 % for serum assays of Estradiol (E2),

luteinizing hormone (LH), follicle-stimulating hormone (FSH) and progesterone, respectively. Samples were assayed in duplicate and all samples were processed in the same assay.

Assay of lipid profile and glucose

The determination of triacylglyceride, total cholesterol, high-density lipoprotein Cholesterol (HDL-Cholesterol), and low-density lipoprotein cholesterol (LDL - LDL-Cholesterol) levels was done using the colorimetric kit (Bio Diagnostic, Egypt, Catalogue No. TR 20-30, CH 12-20, CH 12 30, and CH 12 31, respectively according to the manufacturer's protocols. The estimation of fasting glucose level was done using an enzymatic colorimetric kit (Bio Diagnostic, Egypt, Catalogue No. GL 1320) following the manufacturer's protocols.

Quantitative real-time PCR

Total RNA was extracted from ovarian tissues and blood by using ABTIZOL (applied biotechnology, Egypt). A Nanodrop spectrophotometer (SPECTRO star® Nano (microplate and cuvette Spectrophotometer BMG LABTECH Germany) was used to assess RNA quality and quantity. Then RNA was converted to cDNA via reverse transcription step using ABT H-minus cDNA synthesis kit, (applied biotechnology, Egypt, catalogue number: ABT009) and used according to the manufacturer's instructions. The RNA was added (2000ng). The volume of RNA taken from each sample was calculated (Volume in µl = 2000/conc.) and mixed gently by pipetting of each PCR tube. Obtained RNA concentration was 2000 -2972 ng and the amount of RNA was 0.8 to 1.7 µl. 2000 ng RNA template was added to 2 µl of oligo(dt)18 primer and upto13.5 µl of RNA nuclease-free water was added. Then heating at 65°C for 5 minutes, then the tubes were chilled on ice. Then 0.5 µl of enzyme (H minus MMLV), 4 µl 5X first strand buffer and 2 µl of dNTPs mixture were added. The cycling condition was 42°C for 60 min, then termination of the reaction by heating at 70°C for 5 min. qRT-PCR analysis was performed using the ABT2x qPCR mix kit (Applied Biotechnology, Egypt). The primers were obtained from Sigma Scientific Services Co (Egypt) and designed using the primer Blast program from the National Centre for Biotechnology Information (NCBI) and reconstructed according to the manufacturer's instructions.

Histopathological Examinations

At the end of the experiment, scarification of the anesthetized animals with thiopental was done. Ovarian tissues were harvested from the sacrificed rats. The tissues were fixed in 10% neutral formalin and embedded in paraffin blocks. Sections measuring 5 µ in thickness were obtained, deparaffinized, and stained with hematoxylin and eosin. The sections were cut around the center, along the long axis of the ovary. For histopathological examination by light microscopy (Olympus CX31, Japan). The number of ovarian follicles and corpora lutea in each section were counted digitally using an Axiostar Plus microscope (Carl Zeiss, Thornwood, NY, USA) interfaced with an Axiostar Plus digital camera and Axiovision 4.1 software (Carl Zeiss). The follicles observed were classified into four groups: primordial follicles, primary follicles, secondary (pre-antral) follicles, and Graafian (antral) follicles (Sohrabi *et al*., 2015).

Results

Body weight and ovarian weight

The body weight of the control, HFD, HFD + *S. officinalis*, HFD + bee bread, and HFD/ND groups at the 14th weeks is shown in Fig. 1a, b. The feeding of HFD resulted in a statistically significant increase in final body weight when compared to the control group. On the contrary, the three treated groups showed a statistically significant decline in the final body weight in comparison with the HFD group. The weights of the HFD + *S. officinalis* and HFD + bee bread groups were insignificant compared to the control rats. Collectively, both the control and HFD groups showed statistically significant weight gain. On the other hand, both *S. officinalis* and bee bread treatments drove significant weight loss, while the HFD/ND group resisted weight gain (Fig.1c).

(Fig. 1d) shows the ovarian weight in the five groups. The HFD supplements significantly increased the ovarian weight, whereas *S. officinalis*, bee bread, and shifting to a normal diet supplement could prevent the increase in ovarian weight, especially *S. officinalis*, which significantly decreased the ovarian weight more than bee bread and shifted to normal diet.

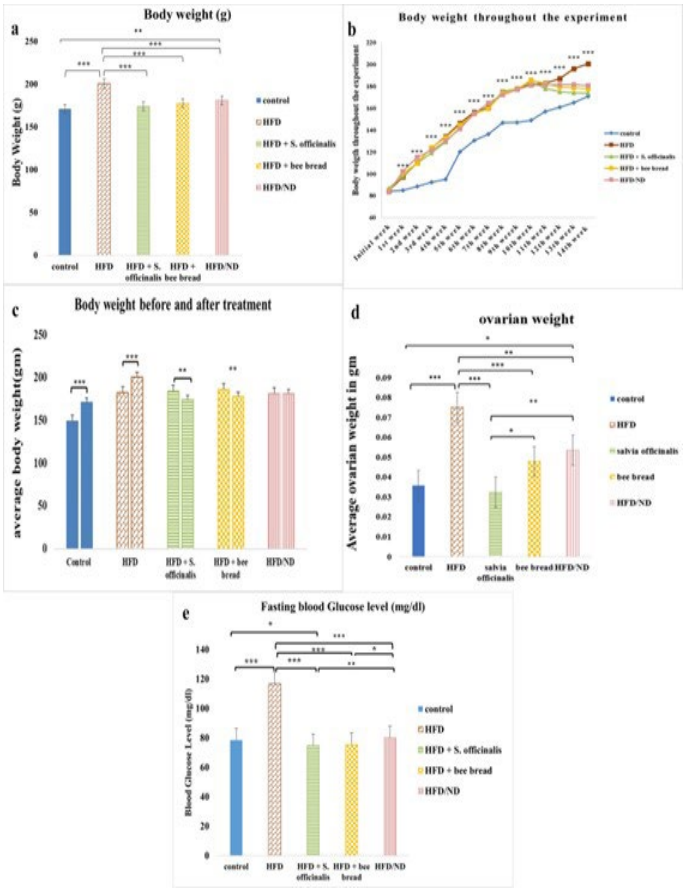

Figure 1. a) Final body weight b) Mean body weight throughout the experiment. c) Average body weight at the 10th week the time before administration of the different treatment according to each group. and at the 14th week the time after treatment d) Ovarian weight in different studied groups at the end of the experiment e) Fasting blood glucose level in different studied groups. at the end of experiment (at the end of the 14th week). Data represent mean±S.E. The one-way ANOVA followed by post hoc test was used for comparison between groups. * P < 0.05, ** P < 0.01, *** P < 0.001.

Fasting blood glucose level and lipid profile

The high-fat diet (HFD) group showed a significant elevation in fasting blood glucose levels in comparison with the control, HFD + *S. officinalis*, HFD + bee bread, and HFD/ND groups. The HFD + *S. officinalis* group showed significantly lower blood glucose than the control group and HFD/ND (Fig. 1 e).

Total cholesterol, triglycerides, and LDL-C were significantly increased by HFD, while HDL-C decreased. The *S. officinalis*, bee bread, and ND supplements normalized the cholesterol and HDL-C levels to similar levels to the control group. Also, the hypolipidemic effect of the three supplements was proved by a decrease in LDL-C and triglyceride levels; however, the HFD+ *S. officinalis* group had significantly lower levels than the HFD+ bee bead and HFD/ND groups, as illustrated in Fig. 2.

Estrus cycle

Table 1 shows the estrus cycle for ten days before the 14th week of the experiment in different studied groups. The HFD group was characterized by a significantly higher percentage of irregular cycles when com-

pared with the control, HFD + *S. officinalis*, HFD + bee bread, and HFD/ND groups. The HFD + bee bread group showed the highest percentage of regular cycles when compared with HFD + *S. officinalis* and HFD/ND administrated groups and had no significant difference with the control group. As regards the cycle frequency, HFD significantly decreased the number of cycles when compared with the control, while the three treated groups showed a nonsignificant increase in the number of cycles when compared with HFD.

HFD group decreased proestrus stage, increased diestrus and diestrus index and resulted in nonsignificant shortening in estrus and metestrus when compared with the control group. *S. officinalis* and bee bread reverse these changes in the estrus cycle. Shifting to a normal diet increased proestrus and decreased diestrus and diestrus index stage insignificantly.

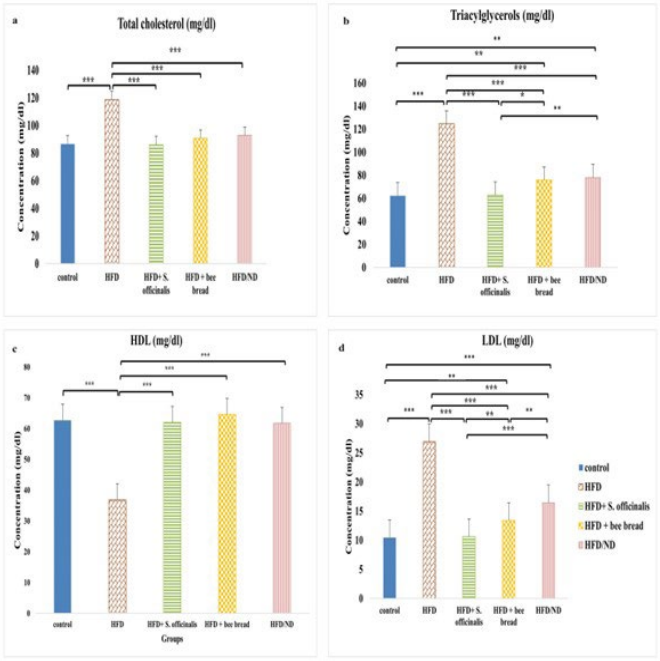

Figure 2. Serum a) total cholesterol, b) triacylglycerols, c) HDL and d) LDL levels in different studied groups at the end of experiment at the end of the 14th week. Data represent mean±S.E. The one-way ANOVA followed by post hoc test was used for comparison between groups. * P < 0.05, ** P < 0.01, *** P < 0.001.

Hormones results

The serum levels of reproductive hormones (estrogen, progesterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH)) in the different studied groups at the end of the experiment (at the end of the 14th week) are illustrated in Table 2. The high-fat diet (HFD) group showed a significant decrease in the estrogen level and a significant elevation in progesterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) in comparison with the control group. The *S. officinalis*, bee bread, and shifting of HFD to a normal diet (HFD/ND) treatment resulted in a statistically significant increase in serum estrogen level and a statistically significant decrease in LH, FSH, and progesterone. Additionally, the HFD+ Bee bread group showed a significant increase in estrogen when compared with the control and HFD/ND groups, a significant decrease in progesterone and LH when compared with the control and HFD/ND groups, and a significant decrease in LH and FSH when compared with the HFD/ND and HFD+*S. officinalis* groups.

Relative mRNA expression of ovarian and blood genes

The TNF-α and IL-6 blood relative mRNA expression levels were significantly increased in the HFD group when compared to the control group. The HFD +S. officials and HFD + bee bread groups showed a significant decrease when compared with the HFD group, but the HFD/ND supplement decreased them insignificantly (Fig. 3 a & b).

Comparing the CYTP 17a relative mRNA expression between the HFD group and the other four supplemented studied groups denotes a significant decrease. The HFD + bee bread group had a higher level than the control group (Fig. 3 c).

The Atg5 relative gene expression in the HFD group showed a significant decrease when compared to control groups. Both *S. officinalis* and bee bread significantly increased the Atg5 relative gene expression when compared with the HFD and HFD/ND groups. While the HFD/ND group showed a significant decrease in comparison with the control, HFD+S. officials, and HFD+ bee bread groups (Fig. 3 d).

There was a significant decrease in the relative mRNA expression of the CCDN1 gene in the HFD group when compared with the control

Table 1. Estrus cycle regularity, Cycle frequency (number of estrus cycles), the duration of the stages of the cycle (proestrus, estrus, metestrus, diestrus), and diestrus index for ten days assessment at the end of the experiment at the 14th week of the experiment in different studied groups.

Group	Regularity		Cycle frequency	Total number of days for each phase				Diestrus index
	Regular	Irregular		Proestrus	Estrus	Metestrus	Diestrus	
Control (n=7)	(6) 85.7%	(1) 14.3%	1.71±0.18	1.71±0.28	3.14±0.40	0.57±0.20	4.57±0.48	45.71±4.81
HFD (n=7)	(1) 14.3%	(6) 85.7%	1.14±0.09 ^{a*}	0.14±0.14 $^{a**, c*, d*, e*}$	2.85±0.40	0.43±0.20	6.57±0.36 $^{a*, c*, d*}$	65.71±3.68 $^{a*, c*, d*}$
HFD + S. officinalis (n=7)	(5) 71.4%	(2) 28.6%	1.50±0.28	1.28±0.28 ^{b*}	3.14±0.70	1.00±0.23	4.57±0.75 ^{b*}	45.71±7.51 ^{b*}
HFD + Bee bread (n=7)	(6) 85.7%	(1) 14.3%	1.64±0.17	1.28±0.42 ^{b*}	3.43±0.53	0.57±0.20	4.71±0.42 ^{b*}	47.14±4.20 ^{b*}
HFD/ ND (n=7)	(3) 42.9%	(4) 57.1%	1.21±0.15	1.14±0.34 ^{b*}	2.43±0.37	0.57±0.29	5.85±0.55	58.57±5.53
P value	0.03		0.16	0.02	0. 682	0.47	0.04	0.04

Data represent mean±S.E. number and percentage significance difference $^{a, b, c, d, e}$ from control, HFD, HFD + S. officinalis, HFD + bee bread, and HFD/ND respectively. Using one-way ANOVA followed by post hock LSD test to compare means of mutable groups, while Chi-Square test used to compare the regularity percentage of the cycle. * P < 0.05, ** P < 0.01.

Table 2. Serum concentration of reproductive hormones in different studied groups at the end of experiment (at the end of the 14th week).

Hormones	Estradiol pg/ml	Progesterone ng/ml	LH MIU/ml	FSH MIU/ml
Control (n=7)	39.74±0.20	13.21±1.10	8.93±0.11	14.32±0.36
HFD (n=7)	31.77±1.70 $^{a ***, c***, d***, e***}$	25.63±0.77 $^{a***, c***, d***, e***}$	11.48±0.41 $^{a***, c***, d***, e***}$	22.85±0.54 $^{a***, c***, d***, e***}$
HFD + S. officinalis (n=7)	43.21±0.52 $^{b ***}$	8.46±0.54 $^{a***, b***}$	6.21±0.36 $^{a***, b***, d**, e**}$	15.42±0.36 $^{b***, d***, e**}$
HFD + Bee bread (n=7)	48.15±2.06 $^{a ***, b***, e**}$	6.42±0.38 $^{a***, b***, e*}$	4.53±0.31 $^{a***, b***, c**, e***}$	10.25±0.98 $^{a***, b***, c***}$
HFD/ND (n=7)	41.25±0.42 $^{b***, d**}$	9.71±0.55 $^{a*, b***, d*}$	8.07±0.27 $^{b***, c**, d***}$	12.37±0.34 $^{b***, c**}$
P value	< 0.001	< 0.001	< 0.001	< 0.001

Data represent mean±S.E. The one-way ANOVA followed by post hoc test was used for comparison between groups. $^{a, b, c, d, e}$ significance difference from control, HFD, HFD + *S. officinalis*, HFD + bee bread, and HFD/ND groups respectively. * P < 0.05, ** P < 0.01, *** P < 0.001

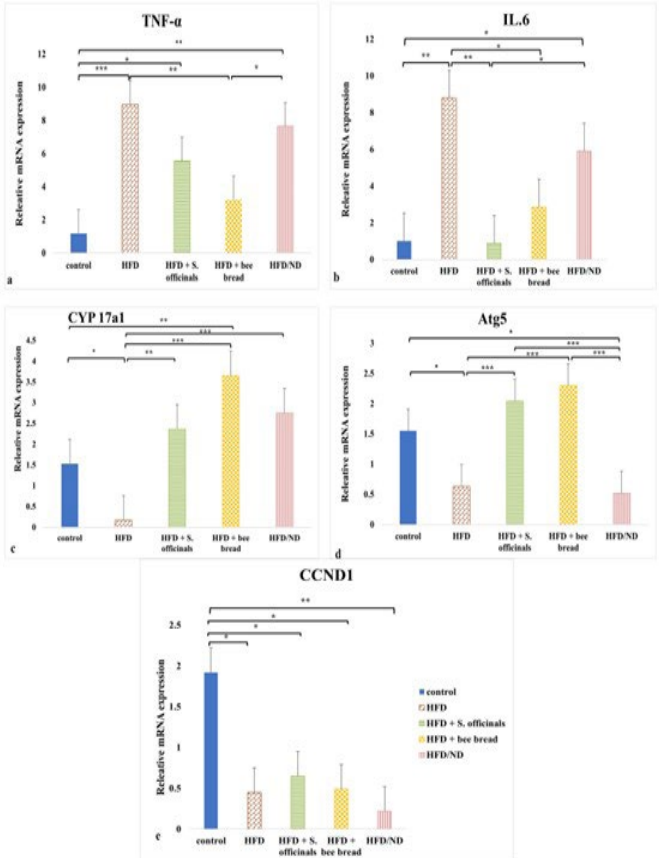

Figure 3. Relative mRNA expression of a) TNF-α, b) IL.6, c) CYP 17a1, d) Atg5 and e) CCND1 in the different studied groups. The Data was presented as mean±S.E. The one-way ANOVA followed by post hoc test was used for comparison between groups. * P < 0.05, ** P < 0.01, *** P < 0.001.

Pathological findings

Histopathological findings

Group I (control negative group)

Microscopic examination of H&E stained tissue sections from the ovaries of the sacrificed rats at the diestrus stage revealed histological features of normal ovarian tissue. The ovarian tissue exhibited a normal central vascular medulla that consists of dense fibrous connective tissue and blood vessels and a compact outer cortex that consists of developing follicles (primordial, primary, secondary follicles), corpus luteum that consist of granulosa lutein cells, supporting stroma, and blood vessels (Fig 4 a).

Group II a (high-fat diet group)

Microscopic examination of H&E stained tissue sections from the ovary of the sacrificed rats at the diestrus stage revealed marked follicular, angiopathic changes, fat vacuoles degeneration and infiltration of inflammatory cells both in periovarian adipose tissue and intraovarian interstitial tissue.

The follicular changes appeared in all 7 examined female rats. These changes were expressed by the appearance of cystic formation (Fig. 4b). The increase of atretic follicles when compared with the control group was also observed in this group. Atretic follicles lined with granulosa layer filled with some deeply stained pyknotic granulosa cells. These deeply stained pyknotic cells had undergone apoptosis with the presence of macrophage cells which phagocytized the apoptotic cells (Fig. 4c, d). Angiopathic changes were manifested by congestion of interstitial blood vessels and the subcapsular blood vessel, which was observed in all 7 examined female rats (Fig. 4e, f). Also, all examined rats' revealed fat vacuoles in the interstitial tissue that appeared in the corpus luteum in 6 rats (Fig. 4g). Infiltration of periovarian adipose tissue with inflammatory cells appeared in 6 female rats of the examined group (Fig. 4 h). In addition, the infiltration of inflammatory cells in the ovarian interstitial tissue was observed in 3 female rats (Fig. 4i).

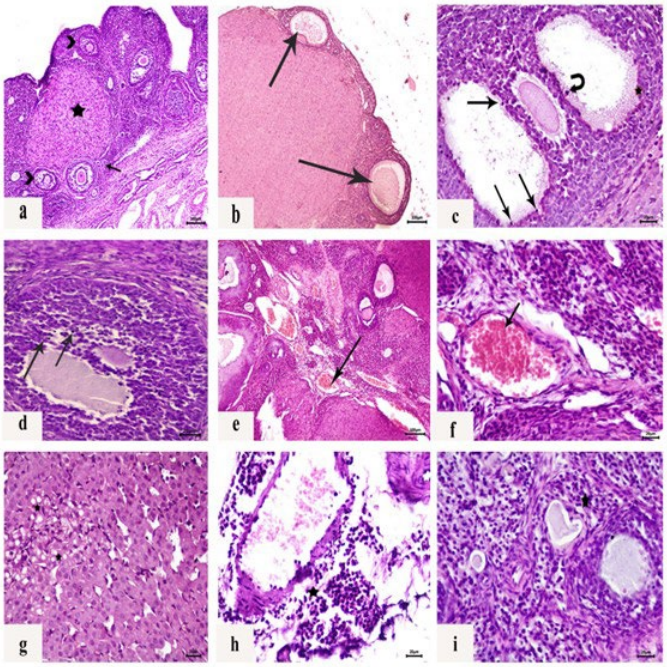

Figure 4. ovary, a) Control negative (ND) administered group showing normal corpus luteum (star), primordial follicles (arrow), primary follicles (arrowhead) (bar= 100 µ). HFD administrated group showing b) cystic formation (arrow), c) Atretic follicles with granulosa layer (star), there were some deeply stained pyknotic granulosa cells (arrow), macrophage cells (curved arrow) which phagocytized the apoptotic cells. d) deeply stained pyknotic granulosa cells (arrow), (bar= 20µ), e) severe congestion in the interstitial blood vessels (arrow) (bar= 100 µ), & f) subcapsular congestion g) clear fat vacuoles in the corpus luteum (star), h) perivascular infiltration of adipose tissue with inflammatory cells (star) (bar= 20µ), and i) intraovarian infiltration of interstitial tissue with inflammatory cells (star) (bar = 20 µ) (H&E).

Table 3. Histopathological score (scale 0 - 3) of the percentage of damage in the ovaries observed by light microscope in the ovary of rats in all experimental groups at the end of the experiment (at the end of the 14th week).

Group	Follicular degeneration	Angiopathic changes		Fat vacuoles appearance	Inflammatory cells Infiltration	
		Hemorrhage	Congestion		Periovarian	Intraovarian
Control (N=7)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
HFD (N=7)	1.21±0.26$^{a***, c **, d*}$	2.50±0.29$^{a***, c ***, d***, e ***}$	2.64±.24$^{a***, c ***, d***, e ***}$	1.71±0.26^{a***}	1.28±0.18$^{a***, c ***, d**}$	0.78±0.28
HFD + *S. officinalis* (N=7)	0.21±0.15^{b**}	0.71±0.18^{b***}	0.43±0.20$^{b ***, d*}$	0.86±0.32	0.21±0.15 ^{b***}	0.21±0.15 ^{e*}
HFD + bee bread (N=7)	0.36±0.18^{b*}	0.64±0.35^{b***}	1.28±0.18$^{a***, b ***, c*}$	1.00±0.28	0.43±0.17 ^{b**}	0.14±0.14 ^{e*}
HFD/ND (N=7)	0.50±0.22	0.50±0.18^{b***}	1.07±0.13$^{a**, b ***}$	0.85±0.26	0.71±0.18 ^{a*}	1.07±0.27$^{a**, c *, d*}$
P Value	< 0.001	< 0.001	< 0.001	0.00	< 0.001	0.00

The data represents mean±S.E. The one-way ANOVA test followed by post hoc was used for compassion between groups. $^{a, b, c, d, e}$ significance difference from control, HFD, HFD + *S. officinalis*, HFD + Bee bread and HFD/ND groups, respectively. * P < 0.05, ** P < 0.01, *** P < 0.001.

GROUP II b (HFD with S. officinalis extract)

The ovary of the HFD with *S. officinalis* extract administered to group 6 female rats revealed marked improvement of all ovarian changes. There was showing increase in the number of healthy follicles with an increase in the number of primordial follicles and the disappearance of cystic formation in 6 rats (Fig. 5a). In the angiopathic changes, only 4 female rats showed mild congestion in interstitial blood vessels accompanied by mild infiltration of interstitial tissue with inflammatory cells in 2 female rats while the 3 female rats showed a disappearance of the congestion (Fig. 5b). The absence of clear fat vacuoles in the corpus luteum was detected in 2 female rats. 3 female rats showed mild clear fat vacuoles in the corpus luteum, and the last 2 rats showed moderate appearance of fat vacuoles (Fig. 5c).

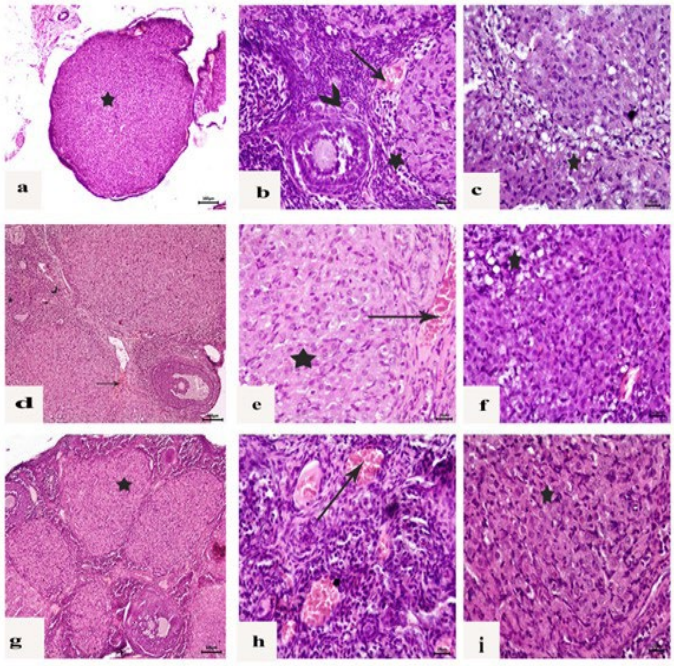

Figure 5. Ovary, HFD with *S. officinalis* extract administered group showing a) absence of hemorrhage and absence of the clear fat vacuoles in the corpus luteum (star) (bar=100µ) b) increased number of primordial follicles (arrowhead) infiltration of interstitial tissue with inflammatory cells (star) mild congestion in the interstitial blood vessels (arrow), and c) presence of the clear fat vacuoles in the corpus luteum (star) (bar=20µ). HFD with bee bread administered group showing d) increased number of primordial follicles (arrowhead), infiltration of interstitial tissue with inflammatory cells (star), mild congestion in the interstitial blood vessels (arrow), (bar=100µ) e) absence of clear fat vacuoles in the corpus luteum (star), mild congestion (arrow) (bar= 20µ), and f) presence of clear fat vacuoles in the corpus luteum (star) (bar= 20µ) (H&E).) HFD/ND administered group showing g) absence of both clear fat vacuoles in the corpus luteum (star) h) congestion in interstitial blood vessels (bar=20µ), i) absence of clear fat vacuoles in the corpus luteum (star) (bar=20µ) (H&E).

GROUP II C (HFD with Bee bread group)

Histopathological examination of the HFD with bee bread administered group showed an increase in the number of healthy follicles with an increase in the number of primordial follicles in 4 female rats (Fig. 5d). In this group, the angiopathic changes improved as 1 female rat showed the absence of congested appearance and 3 female rats presented a mild appearance while the other 3 female rats showed moderate appearance in interstitial blood vessels (Fig. 5e). The absence of fat vacuoles was detected in 2 female rats while 3 female rats exhibited a mild appearance of clear fat vacuoles in the corpus luteum and the other 3 female rats showed a moderate appearance (Fig. 5f). Infiltration of interstitial tissue with inflammatory cells was noticed in 2 female rats while the other 5 female rats showed improvement in inflammatory cell infiltration.

GROUP II d (HFD/ND) (HFD then shifting to normal diet group)

The morphopathological picture of each lesion in The HFD then shifting to the normal diet administered group showed 5 female rats increase in the number of healthy follicles with an increase in the number of primordial follicles and a decrease in the appearance of cystic formation (Fig. 5g). Four female rats revealed mild congestion, and 2 female rats showed a moderately congested appearance in interstitial blood vessels (Fig. 5h). Two female rats showed the absence of clear fat vacuoles in the corpus luteum while 2 female rats still showed clear fat vacuoles (Fig.5i). In this group, the infiltration of interstitial tissue with inflammatory cells in 3 female rats is moderate. The other 2 female rats showed improvement in inflammatory cell infiltration. The histopathological score was presented in (Table 3). The morphometric analysis of ovarian follicles was shown in (Table 4).

Immunohistochemistry

Expressing NFκB is a good marker in immune and inflammatory responses. In the control group, no positive cells were detected (Fig. 6a). Immunohistochemical examination for NFκB in the HFD-administrated group showed severe expression when compared with the control group (Fig. 6b, c). A moderate positive reaction was observed in the HED + *S. officinalis* treated group (Fig. 6d). The HFD + bee bread treated group and (HFD/ND) administered group exhibited mild reactions (Fig. 6e, f).

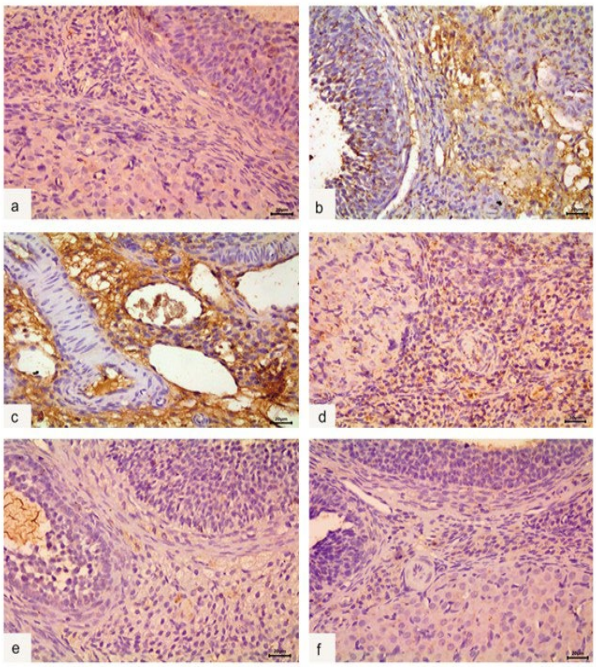

Figure 6. Immunohistochemical staining for NFκB in ovarian tissue showing a) negative reaction in control group. b, c) showing severe positive reaction in the HFD administrated group. d) the HFD + *S. officinalis* treated group show moderate positive reaction. e) the HFD + bee bread treated group showing mild positive reaction and f) the HFD/ND administered group with mild positive reaction (bar=20 µ).

Immunohistochemical examination for caspase-3 in the control group showed a negative reaction (Fig. 7a). A severe positive reaction was seen in the HFD-administered group (Fig. 7b, c). On the other hand, the HED + *S. officinalis* treated group revealed a mild positive reaction (Fig. 7d). While the HFD + bee bread treated group exhibited a moderated positive reaction (Fig. 7e). A mild positive reaction was detected in (HFD/ND) administered group (Fig. 7f).

An examination of the immunoreactivity of $PPAR_{\gamma}$ in ovarian tissue in the present study showed severe positive reactions in the control group (Fig. 8a). However, weak expression of $PPAR_{\gamma}$ was detected in the HFD administrated group (Fig. 8b & c). The HED + *S. officinalis* treated group exhibited a severe positive reaction (Fig. 8d). While the HFD + bee bread treated group showed a moderate positive reaction (Fig. 8e). A mild positive reaction was seen in the HFD then shifting to a normal diet (HFD/ND)

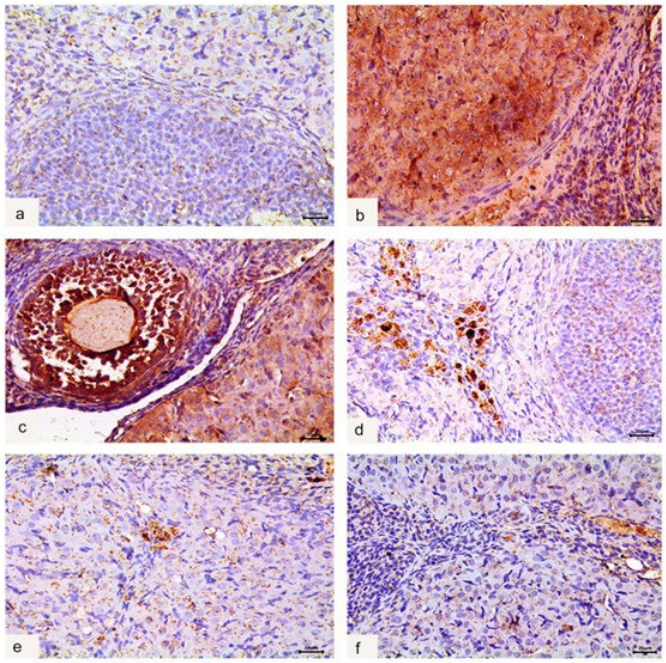

Figure 7. Immunohistochemical staining for caspase-3 in ovarian tissue showing a) negative reaction in control group. b, c) showing sever positive reaction in the HFD administrated group. d) the HFD + *S. officinalis* treated group show mild positive reaction. e) the HFD + bee bread treated group showing moderate positive reaction and f) the HFD/ND administered group with mild positive reaction (bar=20 µ).

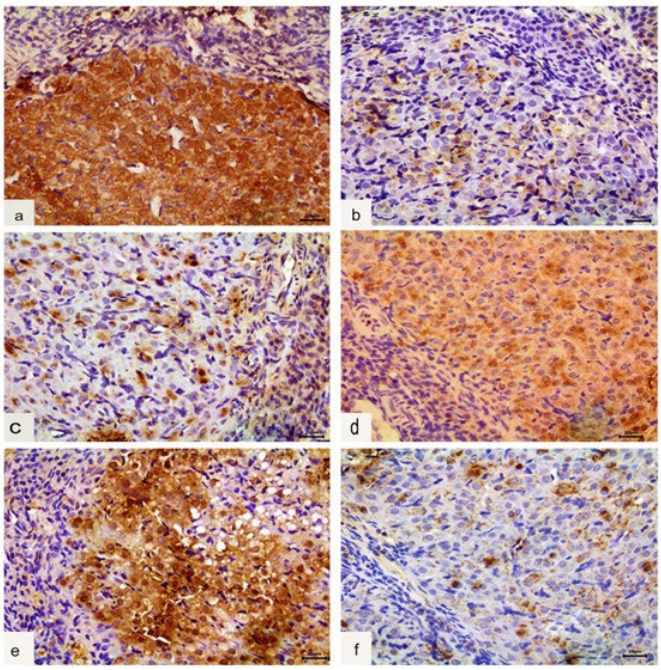

Figure 8. Immunohistochemical staining for $PPAR_{\gamma}$ in ovarian tissue showing a) mild positive reaction in control group. b, c) showing sever positive reaction in the HFD administrated group. d) the HFD + *S. officinalis* treated group show moderate positive reaction. e) the HFD + bee bread treated group showing mild positive reaction and f) the HFD/ND administered group with mild positive reaction (bar=20 µ).

Table 4. Morphometric analysis of the number of different follicles, and cystic follicles in the different studies group at the end of the study at the 14th week.

Group	Primordial follicles	Primary follicles	Secondary follicles	Tertiary follicles	Cystic follicles
Control	13.57±2.54	1.85±0.26	1.85±0.26	1.71±0.28	1.29±0.52
HFD	2.85±1.24 $^{a***, d*}$	4.43±0.99 $^{a**, c***, d***}$	3.42±0.61 $^{a**, c***, d***, e*}$	3.57±0.61 $^{a**, c***, d***, e***}$	3.86±0.59 $^{a***, c**, d***, e***}$
HFD + *S. officinalis*	6.57±1.15 ^{a**}	0.71±0.28 $^{b***, e**}$	1.28±0.42 ^{b***}	0.85±0.34 ^{b***}	1.43±0.37 ^{b**}
HFD + bee bread	8.71±1.06 $^{a*, b*}$	0.85±0.14 $^{b***, e**}$	1.14±0.14 ^{b***}	0.85±0.55 ^{b***}	1.00±0.57 ^{b***}
HFD/ND	6.00±1.31 ^{a**}	3.28±0.78 $^{d**, c**}$	2.14±0.34 ^{b*}	0.57±0.29 ^{b***}	1.00±0.30 ^{b***}
P value	< 0.001	< 0.001	0.00	< 0.001	< 0.001

The data represents mean±S.E. The one-way ANOVA test followed by post hoc was used for compassion between groups. $^{a, b, c, d, e}$ significance difference from control, HFD, HFD + *S. officinalis*, HFD + Bee bread and HFD/ND groups, respectively. * P < 0.05, ** P < 0.01, *** P < 0.001.

Table 5. Immunohistochemical scoring (scale 0 - 3) of the percentage of the reaction in each of NF-κB, PPAR*γ* and caspase 3 in the ovaries observed by light microscope in the ovary of rats in all experimental groups at the end of the experiment (at the end of 14th week).

Group	NF-κB	Caspase 3	PPAR*γ*
Control (n=7)	0.43±0.202	0.28±0.18	3.00±0.00
HFD (n=7)	3.00±0.000 $^{a***, c***, d***, e***}$	3.00±0.00 $^{a***, c***, d***, e***}$	1.00±0.000 $^{a***, c***, d***}$
HFD + *S. officinalis* (n=7)	1.29±0.184 $^{a***, b***}$	0.86±0.14 $^{a*, b***, d*}$	2.86±0.143 $^{b***, d***, e***}$
HFD + bee bread (n=7)	0.86±0.143 ^{b***}	1.43±0.20 $^{a***, b***, c*}$	2.00±0.218 $^{a***, b***, c***, e**}$
HFD/ND (n=7)	0.86±0.143 ^{b***}	1.142±0.14 $^{a***, b***}$	1.29±0.184 $^{a***, c***, d**}$
P value	< 0.001	< 0.001	< 0.001

The data represents mean±S.E. The one-way ANOVA test followed by post hoc was used for compassion between groups. $^{a, b, c, d, e}$ significance difference from control, HFD, HFD + *S. officinalis*, HFD + Bee bread and HFD/ND groups respectively. * P < 0.05, ** P < 0.01, *** P < 0.001.

administered group (Fig. 8f). Scoring of expression of NFκB, caspase-3 and $PPAR_{\gamma}$ in different experimental groups demonstrated in (Table 5).

Discussion

According to the current findings, the HFD supplement led to an irregular estrus cycle which is manifested by longer diestrus phases and reduced proestrus phases. These alterations are attributed to the shortening of the follicular phase through early selection and maturation of dominant follicles

which may be due to low levels of estradiol (Kannan *et al.*, 2019). Also, it has been proposed that overstimulation of insulin signalling in the ovary, as well as alterations in proliferator-activated receptor γ (PPARγ) actions, may be involved in the disruptions of the estrous cycle with HFD feeding (Hohos and Skaznik-Wikiel, 2017).

The HFD-supplemented groups with *S. officinalis* and bee bread showed improvements in estrous cycle regularity. This improvement in the estrous cycle could be attributed to the estrogenic activity of *S. officinalis* (Al-Ani *et al.*, 2020) and the antioxidant activity of bee bread due to the presence of active phenolic compounds and α-tocopherol (Othman *et al.*, 2019) and (Borycka *et al.*, 2015).

The present histopathological results revealed an increase in the number of primordial follicles and a decrease in atretic follicles in the bee bread-administered group in comparison to HFD-fed rats. Bee bread has a positive impact on ovarian folliculogenesis due abundance of nutrients as minerals, vitamins, lipids, amino acids, enzymes, and fatty acids includ-

ed in bee bread may increase the ovulatory follicles (Karaman, 2019) and also the glucose content will promote follicular growth and inhibits follicular atresia (Bakour *et al*., 2019) because it can be used as major energy production by ovarian follicular cells (Tri Widayati *et al*., 2018). To a similar extent, the bee bread supplement to HFD-fed male rats protected them from infertility (Suleiman *et al*., 2021).

This irregularity in the estrous cycle parallels the disturbance in reproductive hormones. Our results showed that HFD consumption resulted in a significant decrease in estradiol levels, which was consistent with the Fernandez *et al*. study that reported HFD decreased aromatase expression and estradiol levels (Fernandez *et al*., 2017; Ma *et al*., 2016). Additionally, a significant increase in serum LH, FSH and progesterone levels in the HFD-fed group in comparison with the control group. This can be explained by hypergonadotropism (Akamine *et al*., 2010) and altering folliculogenesis (Hohos and Skaznik-Wikiel, 2017).

The *S. officinalis*-treated group showed a significant increase in estradiol and a decrease in LH, FSH, and progesterone serum levels when compared to the HFD-fed group. This could be attributed to inducing estrogen synthesis (Sabry *et al*., 2022) which in turn reduced the serum levels of LH and FSH through a negative feedback mechanism (Imade *et al*., 2018). These present effects are matched with those of the use of 200 mg of S officinalis in obese rats (Sabry *et al*., 2022). Similarly, the bee bread administrated group showed a significant increase in the level of estradiol, and a decrease in LH, FSH, and progesterone when compared with the fed group as mentioned above bee bread is a potent antioxidant due to its richness with phenolic compounds (Othman *et al*., 2019) and exerts anti-inflammatory action (Hämäläinen *et al*., 2007).

In our study, the mRNA level of the CYP17A1 gene in ovarian tissue was significantly decreased in the HFD-fed group when compared to the control group. A previous study reported a decrease in CYP17A1 gene expression in obesity (Tabur *et al*., 2016). In contrast to our study, the ovaries of female rats fed HFD were previously reported to have an increased level of CYP17A1 (Long *et al*., 2022). It has been observed previously that autophagy insufficiency hinders the differentiation markers of GCs. Results in decreased levels of steroidogenic enzymes (Shao *et al*., 2022). As recorded in previous studies, HFD downregulates autophagy (Koga *et al*., 2010). Together, this finding can explain why HFD downregulates the level of mRNA of CYP17A1 in the ovaries of HFD-induced obese rats.

In the ovary, Atg5 is important for normal follicular development. Its downregulation results in an increase in atretic follicles, which may indicate ovarian dysfunction (Gawriluk *et al*., 2014). Our findings showed that the mRNA level of Atg5 was significantly decreased in the ovarian tissue of HFD-fed rats. This is consistent with a high-fat diet (HFD, 60% kcal in fat) altered membrane lipid composition and downregulated autophagy by reducing autophagosome/lysosome fusion (Koga *et al*., 2010). Also, it could be explained that obesity induces stimulation of mTOR signalling, which in turn suppresses autophagy (Galluzzi *et al*., 2014).

S. officinalis l., bee bread administration, and shifting to a normal diet resulted in upregulation of the mRNA level of Atg5 in ovarian tissue. This is in agreement with the previous study, which revealed that *S. officinalis* extract increased the expression of Atg5 in ovarian tissues (Alrezaki *et al*., 2021). This could be explained due to its component, apigenin which alleviated LPS-induced myocardial injury by modulating autophagic components, including Atg5 (Li *et al*., 2017). Whereas the bee bread component, kaempferol was found to be involved in the autophagic cycle, elevating the protein levels of Atg 5, Atg 7, and Atg 12 (Kiruthiga *et al*., 2020).

Cell proliferation is important for controlling folliculogenesis in the ovary. CCND1, as mentioned previously, has a central role in promoting entry into the cell cycle and plays a critical role in the decision of a cell to continue proliferating (Yang *et al*., 2006). HFD-induced obesity results in ROS elevation (Sun *et al*., 2020) which in turn induces cell cycle arrest or cell death (Lander *et al*., 1995; Chua *et al*., 2009). This is in line with the present result as the HFD-fed group revealed a significant decrease in the CCND1mRNA level when compared with the control group. CCND1 decrease in the ovaries of mice fed indicated that HFD-induced obesity results in cell cycle arrest causing defective folliculogenesis and ovarian dysfunction (Wu *et al*., 2015). The administration of *S. officinalis* extract, bee bread, and shifting from HFD to a normal diet revealed a nonsignificant increase in the CCND1 mRNA level when compared to the HFD group.

The HFD-induced ovarian dysfunction could be confirmed by the histopathological picture of the ovaries. Our results showed that the HFD group revealed significantly impaired folliculogenesis that was expressed in an increased number of both atretic follicles and growing follicles, fewer primordial follicles, and a decreased corpus luteum in the HFD group in comparison to the control group. Our finding was in agreement with previous studies that demonstrated that HFD increases the number of arthritic follicles (Wang *et al*., 2014). Another study revealed that HFD resulted in increased anovulation and decreased ovulation, and their ovarian cells showed signs of lipid buildup (Wu *et al*., 2010). This is in agreement with our study, as the HFD group showed the presence of fat vacuoles, and the appearance of cystic follicles was significantly increased in the HFD group in comparison to the control group. In contrast, previous studies reported no effect of HFD consumption on the number of primary follicles, secondary follicles, antral follicles, atretic follicles, or corpora lutea when compared with a standard diet (Hilal *et al*., 2020).

Angiopathic changes and inflammatory cellular reaction was significantly increased either interstitially or within the corpus luteum in the HFD group in comparison with the control group. Our findings are consistent with the study that demonstrated that HFD, regardless of obesity, negatively mediates the increased local inflammation characterized by increased macrophage infiltration in the ovaries of animals fed HFD (Skaznik-Wikiel *et al*., 2016).

S. officinalis + HFD, bee bread + HFD-treated groups exhibited a significant increase in the number of healthy follicles and primordial follicles and increased the number of corpora luteum. Also, cystic follicle disappearance was observed in both treated groups. The hemorrhage was significantly decreased in the *S. officinalis* + HFD, bee bread + HFD, and HFD/ND groups. Also, the congested appearance was improved in this treated group with a special good response to *S. officinalis* administration.

The improvement of folliculogenesis with *S. officinalis* administration could relate to the diterpenoids that represent the second major component of the sage plant, as well as 13-epimanol and carnosol (Ruan *et al*., 2017). In addition to the anti-inflammatory and antioxidant activity of *S. officinalis* mentioned in this study, *S. officinalis* L. was reported to have estrogenic activity (Sabry *et al*., 2022). Also, in our study, we observed that *S. officinalis* L. significantly decreased the inflammatory reaction and angiopathic changes induced by HFD feeding. It could be related to anti-inflammatory compounds (Albano and Miguel, 2011).

Bee bread exerts a beneficial effect on ovarian folliculogenesis as it maintains follicular growth and prevents follicular atresia. The bee bread + HFD-treated group showed a significant decrease in abnormal follicles, a significant increase in healthy follicles, and an increase in ovarian reserve. This activity could be explained as bee bread has a rich nature of minerals, vitamins, lipids, amino acids, enzymes, and fatty acids that were reported to improve the number of ovulatory follicles and ovulation rate that was observed previously in cycling goats (Karaman, 2019). Bee bread was observed in our study to improve angiopathic changes, as it was observed that the bee bread + HFD group showed a significant reduction in hemorrhagic appearance and low congestion in ovarian tissue. This could be related to the richness of bee bread with polyphenolic compounds, as mentioned, and vitamin C (Olaniyan *et al*., 2019).

HFD feeding resulted in suppressed expression of PPARγ in ovarian tissue as our results recorded a decrease in PPARγ immunohistochemical expression in the HFD administrated group. Our finding agrees with the previous study, which reported that HFD has a negative impact on the level of PPARγ as HFD lowers the PPARγ level significantly (Olaniyi *et al*.,

2023). Despite another study revealing that PPARγ was found to be not affected in HFD-fed mice (Minge *et al*., 2008). The decreased expression level of PPARγ in obesity could be explained as obesity and high-fat diet inducing inflammation and cleavage of the p35 protein to generate p25. The p25 activates CDK5, which phosphorylates PPARγ thereby preventing the transcription of specific PPAR-γ targets (Wei *et al*., 2012). Our work revealed the LH level increased. This could also explain the downregulation of PPARγ expression. It was demonstrated that expression of PPARγ is downregulated in the ovary in response to the LH surge. Disrupting the expression of PPARγ in the ovary, therefore, could potentially affect oocyte developmental competence.

Polyphenols from natural products are reported to activate PPARγ receptors by increasing their expression (Rzepecka-Stojko *et al*., 2017). Our results showed that the administration of *S. officinalis* in the HFD + *S. officinalis*-treated group showed severe immunohistochemical expression of PPARγ in the ovaries. This result was consistent with a previous study that reported that *S. officinalis* extract activates PPARγ (Ghorbel *et al*., 2020).

The HFD + bee bread-administered group exhibited a moderate increase in immunohistochemical staining of PPARγ in ovarian tissue. This is related to the richness of bee bread with phenolic compounds. As reported, quercetin activates PPARγ receptors by increasing their expression (Rzepecka-Stojko *et al*., 2017). While shifting to a normal diet after HFD feeding, the HFD/ND group showed mild immunohistochemical expression of PPARγ in ovarian tissue. This indicated that the changing diet was not enough to enhance PPARγ expression and its exact function in the ovaries.

In our study, HFD feeding was found to induce severe immunohistochemical expression of caspase-3 in ovarian tissue in comparison with the control group. Our results are in consistent with a previous study that showed a significant increase in caspase-3 expression in the ovary in rats feeding HFD for 9 weeks (Hussain *et al*., 2016). HFD-induced obesity causes excessive apoptosis of the GCs of the ovaries which causes abnormal follicular development and ovarian function failure (Wu *et al*., 2015). HFD feeding induces apoptosis due to intracellular lipid accumulation in many tissues, eliciting lipotoxicity responses that activate stress pathways that can culminate in apoptosis (Robker *et al*., 2011).

Our result revealed that the *S. officinalis* + HFD treated group showed mild immunohistochemical reaction positive reaction in ovaries when compared with the HFD administrated group. This revealed that *S. officinalis* exhibited antiapoptotic activity (Taeimouri and Rahimi, 2022) due to the direct antioxidant and anti-inflammatory action of *S. officinalis*, reduction of ROS production and DNA damage, which prevented the increase of p53 levels. On the contrary, high doses of more than 500 µg/ml can induce apoptosis (Monsefi *et al*., 2017).

The bee bread + HFD and HFD/ND treated groups showed moderate positive caspase-3 expression immunohistochemical reactions. These results for bee bread were supported by the previously reported suppression of caspase-3 expression in HFD-induced obese rats. This may be due to the ability of bee bread to decrease oxidative stress and inflammation (Suleiman *et al*., 2020; Othman *et al*., 2022).

Conclusion

S. officinalis and bee bread alleviate HFD-induced ovarian dysfunction through improvements in folliculogenesis, estrous cycle, reproductive hormone levels, and the expression of genes related to steroidogenesis and the cell cycle. Furthermore, both *S. officinalis* aqueous extract and bee bread have anti-obesity, hypolipidemic, anti-inflammatory, and anti-apoptotic effects. Shifting from HFD to a normal diet to some extent improved the histopathological changes induced by HFD feeding. Despite supplementation of both bee bread and *S. officinalis* with HFD, they showed results that outperformed the shift from HFD to a normal diet. This indicates that both bee bead and *S. officinalis* might be potent natural therapeutic agents for ovarian dysfunctions.

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Conflict of interest

The authors have no conflict of interest to declare.

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