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Research Article

MODULATORY EFEECTS OF TRIFOLIUM PRETENSE EXTRACT AND ROYAL JELLY ON THE FUNCTION OF HYPOTHALAMIC-PITUITARY-OVARIAN AXIS IN OVARIECTOMIZED RATS

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ABSTRACT

Objective: This study was designed to investigate the potential estrogenic effects of phytoestrogen *Trifolium pretense* (red clover) and worker honey bee secretion (Royal jelly) on ovariectomized rat as a model of postmenopausal disorder.

Methods: The animals were divided into 4 groups: 1- sham-operated group (Sham), 2- ovariectomized (OVX) group, 3- OVX treated orally with red clover (750mg/kg/day), 4- OVX treated orally with Royal jelly (750mg/kg/day). Uterine weight, Serum estrogen, follicular stimulating hormone (FSH), luteinizing hormone (LH)), total cholesterol, triglyceride and glucose were estimated. Neurotransmitters determination for serotonin (5-HT) and dopamine (DA) were also assayed in hypothalamus.

Results: The ovariectomy caused significant reduction in uterine weight ($p<0.05$), as well as reduction in serum estrogen content and hypothalamic content of 5-HT and DA, whereas, it caused significant elevation in level of LH, FSH, total cholesterol, triglyceride and glucose in serum at $p<0.05$. The treatment with red clover and Royal jelly stimulated the uterine weight gain as a result of increase in serum estrogen level, they diminished the elevated serum FSH and LH and significantly decreased the triglycerides levels in spite of being caused no significant change in total cholesterol and glucose level. In addition, they regulate the hypothalamic neuronal function by elevation of 5HT and DA secretion as compared to OVX group.

Conclusion: The results of the current study revealed that, red clover extract and Royal jelly were capable of acting at multiple targets which probably could improve function of hypothalamic-pituitary-ovarian axis in OVX rats mimicking symptoms as observed in postmenopausal females.

Keywords: Trifolium pretense; Royal jelly; Ovarian hormone; Pituitary hormones; Neurotransmitters; OVX rats.

INTRODUCTION

In climacteric and premenopausal women, low serum levels of 17β-estradiol (E2) often result in symptoms such as hot flashes or degenerative processes such as osteoporosis. This has prompted women to receive hormone replacement therapy (HRT) to prevent these ageing-associated symptoms or diseases [1]. Menopausal disorders are conventionally treated with synthetic estrogens. However, conventional hormone replacement therapy is suspected to increase the risk of breast cancer and to cause other undesirable side effects, such as breast tenderness and uterine bleeding [2]. Because of these undesirable side effects, many women seek more natural alternatives, such as the so-called phytoestrogens. These alternatives include soybean products that contain estrogen-like molecules such as daidzein and genistein. Soybeans and soybean-derived products have recently become common additives in health foods and beverages. Phytoestrogens are similar to mammalian estrogens both structurally and functionally, and are considered to prevent menopausal symptoms [3, 4]. As a result, natural estrogenic alternatives for the treatment of menopausal pathologies and symptoms are frequently considered, because this offers the hope of improved safety and greater compliance.

The estrogenic compounds isolated from red clover are isoflavones, including genistein, daidzein, biochanin A and formononetin [5]. Two of these compounds, genistein and daidzein, are also found in soy and have been reported to cause an increase in uterine weight [6]. Previous studies have indicated that red clover extracts induce estrogen-responsive proteins, up-regulate the expression of the estrogen-inducible genes progesterone receptor and presenilin 2 and contain ligands, which compete for 17β-estradiol with both estrogen receptors (ERα and ERβ) [7].

Similarly, royal jelly (RJ) has become a common additive in health foods and beverages. RJ is a viscous substance secreted by the hypopharyngeal and mandibular glands of worker honeybees (*Apis mellifera*) and is an essential food for both the queen and her larvae. RJ is a mixture of many constituents including proteins, free amino acids, lipids, vitamins and sugars [8]. 10-hydroxy-trans-2-decenoic acid is a characteristic constituent of RJ. It has been reported to exhibit physiological and pharmacological effects in mammals, including vasodilative and hypotensive activities, antihypercholesterolemic activity and anti-inflammatory functions. In addition to these activities, RJ has been suggested to improve menopausal symptoms. It has been shown to alleviate the so-called autonomic imbalance in menopausal women, leading to the hypothesis that RJ or its components have effects on gene expression similar to, or mimic the effects of estrogen [9].

In the present study, a well-developed experimental model of estrogen deficiency induced by ovariectomy (OVX) in middle-aged female rats, which was expected to exhibit the similar systemic symptoms as observed in pre or postmenopausal women was obtained. The research was to explore the possible mechanisms underlying the pharmacological action of red clover and royal jelly by measuring the levels of several menopausal-associated pituitary–ovarian hormones changes (namely estrogen, FSH and LH) as well as the level of serum cholesterol, triglycerides and glucose. In addition, the monoamine-releasing effect of both subjects in rat hypothalamus (DA and 5-HT) as indices of dopaminergic and serotonergic activity, were determined.

MATERIALS AND METHODS

Materials

Fresh royal jelly was purchased from faculty of agriculture, Cairo University, Cairo, Egypt. The animals daily received oral administration of the secretion at a dose of (750mg/kg. b.wt) according to Kridli and Al-khetib, [10]. Trifolium pretense (red clover) extract (PSC0319) was purchased from Linnea SA, Switzerland. The animals daily received oral administration of the extract at a dose of (750 mg/kg) according to Joanna *et al.* [11].

Animals and treatments

Forty adult female rats (120–150 g) were obtained from the holding company for biological products and vaccines (VACSERA) Cairo, Egypt. They were housed in polypropylene cages maintained in the Laboratory of Physiology, Faculty of Science, Helwan University under normal environmental conditions of temperature, humidity and light. They were allowed to feed on a standard pelled diet (ad libitum). The animals were kept for about one week before experimentation to adapt to the laboratory conditions. They were randomized into four groups of ten rats

each. Three groups were bilaterally ovariectomized (OVX) under pentobarbital sodium anesthesia (50 mg/kg i.p.) [12] and one group was sham-operated (SHAM). Two groups of the OVX animals were treated orally with either red clover (750mg/kg/day) or Royal jelly (750mg/kg/day) once daily for 8 weeks post-ovariectomy. At the end of the experiments, 8 animals from each group were suddenly decapitated; the serum, uteri and brain were collected.

Determination of uterine weight

The uteri were collected, trimmed of fat and connective tissue, cut open and drained of intrauterine fluid and weighed.

Endocrinological analysis

Determination of (FSH) and (LH) by enzyme-linked immunosorbent assay (ELISA) kits according to Rose [13] and Rebar et al. [14] respectively; and estradiol determination of total E2 by radioimmunoassay (RIA) kits according to Xing et al. [15].

Determination of 5-HT and DA

Brain samples were excised very quickly from the cranial cavity within 30 sec after decapitation. The fresh brains were dipped in chilled saline (0.9% w/v); the hippocampus was isolated and stored at low temperature (−70°C) until analysis of 5-HT and DA. The first step in determination of the brain monoamine by HPLC method involved weighing and homogenization of the tissue in 1/10 weight/volume of 75% aqueous HPLC grade methanol. The homogenate was spun at 3000 r.p.m. for 10 min and the supernatant was used for monoamine determination immediately extracted from the trace elements and lipids by the use of solid phase extraction CHROMABOND column NH2 phase Cat. No. 730031. The sample was then injected directly into an AQUA column 150 54.6 mm 5 µC18, purchased from Phenomenex, USA under the following conditions: mobile phase 97/3 20 Mm potassium phosphate, pH 3.0/methanol, flow rate 1.5 ml/min, UV 270 nm. Dopamine and serotonin were separated after 12 minutes. The resulting chromatogram identified each monoamine position and concentration from the sample as compared to that of the standard, and finally, the determination of the content of each monoamine as µg/gram brain tissue [16].

Serum total cholesterol, triglyceride and glucose

Serum concentrations total cholesterol and glucose were determined using commercial enzymatic colorimetric kits (Diamond Diagnostics, Egypt) according to method of Richmond, [17] and Sharma et al. [18] respectively. Serum triglyceride concentrations were determined using Commercial kit (BioSystems S.A. Costa Brava 30, Barcelona, Spain) according to method of McGowan et al. [19].

Statistical analysis

The experiment was set up with a completely randomized design. Data were presented as means±S.E for the indicated number of independently performed experiments using the SPSS package (SPSS 17.0 for Windows). The statistical significances within parameters were evaluated by one-way and multiple analysis of variation (ANOVA), where significant differences at $P<0.05$.

RESULTS

Effect of red clover and Royal jelly on ovariectomy-induced alternations in uterine weight

The OVX resulted in marked decrease in the uterine weight (-50.6%) at $p<0.001$ (Table 1). Daily oral administration of red clover after 10 days of ovariectomy for 8 weeks resulted in a significant increase in uterine weight (52.97%) compared to OVX group ($p<0.05$) and it still significantly decreased as compared to sham group (24.6%). Similarly, the oral administration of Royal jelly (750 mg/kg/day) for 8 weeks started after 10 days of the operation showed also a significant elevation in uterine weight as compared to OVX group in spite of being significantly decreased as compared to sham group ($P<0.05$).

Table 1: Effect of red clover and Royal jelly on ovariectomy-induced alternations in uterine weight

	Uterine weight (mg)
SHAM	410.4±9.13
OVX	202.5±4.65a
% change	(-50.6%)
OVX + red clover	309.2±4.62ab
% change	(-24.6%)
OVX + Royal jelly	294.7±6.65ab
% change	(28.2%)

- Data are mean ± SE of 8 rats.

- (a) significant different from SHAM group at $p < 0.05$.

- (b) Significant different from OVX group at $p < 0.05$.

- (%) percentage change from SHAM group.

- Abbreviation: SHAM=sham operated group; OVX=ovariectomized; OVX + red clover= OVX group treated orally with red clover (750mg/kg/day) and OVA + Royal jelly= OVX group treated orally with royal jelly (750mg/kg/day).

Effect of red clover and Royal jelly treatment on serum ovarian hormone (estrogen)

As shown in Table 2, the level of serum estrogen in sham operated group recorded (605±.5.67 Pg/ml). As a result of OVX, the estrogen level in serum was significantly reduced (290±5.43 Pg/ml) at $p<0.05$ with percentage change of (-52.1%) as compared to sham group. The daily treatment with red clover for 8 weeks resulted in a significant increase in serum estrogen (93.1%) as compared to OVX group, while, it showed a significant decrease as compared to sham group (- 12.4%). In the same manner, the administration of royal jelly also, caused a significant elevation in serum estrogen level as compared to OVX rats (460±3.56 Pg/ml) but as compared to Sham group, the estrogen level still significantly reduced at $p<0.05$.

Effect of red clover and Royal jelly treatment on serum pituitary hormones (LH and FSH)

The secretions of LH and FSH in OVX group ascended following ovariectomy procedure recording (6.4±0.99 MLU/ml and 8.4±0.43 MLU/ml) respectively at $p<0.05$ as compared to sham operated group that recorded LH (3.8±0.46 MLU/ml) and FSH (4.87±0.32 MLU/ml). The 8-weeks treatment of OVX rats with red clover and royal jelly did not affect the serum levels of both pituitary hormones (FSH and LH) as compared to OVX group (Table 3).

Table 2: Effect of red clover and Royal jelly treatment on serum ovarian hormone (estrogen)

	Estrogen (Pg/ml)
SHAM	605.2±.5.67
OVX	290.01±5.43a
% change	(52.1%)
OVX + red clover	530.6±7.65ab
% change	(-12.3%)
OVX + Royal jelly	460.5±3.56ab
% change	(-23.9%)

- Data are mean ± SE of 8 rats.

- (a) Significant different from SHAM group at $p < 0.05$.

- (b) Significant different from OVX group at $p < 0.05$.

- (%) percentage change from SHAM group.

- Abbreviation: SHAM=sham operated group; OVX=ovariectomized; OVX + red clover= OVX group treated orally with red clover (750mg/kg/day) and OVA + Royal jelly= OVX group treated orally with royal jelly (750mg/kg/day).

Effect of red clover and Royal jelly treatment on Hypothalamus monoamines (DA and NE)

As shown in Table 4, the ovarian removal in OVX group resulted in a significant decrease in both 5-HT and DA in hypothalamus recording -20.53% and -27.12% respectively as percentage change from sham group. The oral administration of red clover

for 8 weeks in OVX rats resulted in significant elevation in serotonin content in hypothalamus as compared to OVX group while it recorded significant reduction as compared to sham group ($p<0.001$). Also, red clover treatment caused a significant increase in the reduced DA measured in OVX group in spite of being showed no significant changes as compared to sham group. On the other hand, the treatment with royal jelly recorded a significant increase in 5-HT content ($p<0.001$) as compared to OVX group while no significant changes was observed in DA content due to royal jelly treatment as compared with both OVX and sham group.

Effect of red clover and Royal jelly treatment on Serum total cholesterol, triglyceride and glucose

Table 5 recorded the value of the serum content of (total cholesterol, triglyceride and glucose) studied in current investigation. The OVX resulted in significant increase ($p<0.05$) in the serum total cholesterol (13.7%), triglyceride (20.3%) and glucose (17.6%) as compared to sham operated group at $p<0.05$. Daily oral administration of red clover and royal jelly for 8 weeks resulted in significant decrease in triglyceride in serum recording (115.3±3.97 mg/dL and 117.8±5.76 mg/dL) respectively as compared to OVX group in spite of being no significant change was observed as compared to sham group. On the other hand, the treatment with both extracts did not affect the elevated serum total cholesterol measured in OVX rats. However, the treatment with Royal jelly resulted in no significant changes were observed in serum glucose as compared to sham operated group while it was significantly decreased as compared to OVX group (136.6±6.13 mg/dL).

Table 3: Effect of red clover and Royal jelly treatment on serum pituitary hormones (LH and FSH)

	FSH (MLU/ml)	LH (MLU/ml)
SHAM	4.87±0.32	3.8±0.46
OVX	8.4±0.43a	6.4±0.99a
% change	(75.0%)	(68.4%)
OVX + red clover	5.91±0.64	5.01±0.96
% change	(22.9%)	(31.8%)
OVX + Royal jelly	6.14±0.54	5.22±0.12
% change	(26.9%)	(37.3%)

- Data are mean ± SE of 8 rats.

- (a) Significant different from SHAM group at $p < 0.05$.

- (b) Significant different from OVX group at $p < 0.05$.

- (%) percentage change from SHAM group.

- Abbreviation: SHAM=sham operated group; OVX=ovariectomized; OVX + red clover= OVX group treated orally with red clover (750mg/kg/day) and OVA + Royal jelly= OVX group treated orally with royal jelly (750mg/kg/day).

Table 4: Effect of red clover and Royal jelly treatment on Hypothalamus monoamines (DA and NE)

	Serotonin (µg/gm tissue)	Dopamine (µg/gm tissue)
SHAM	1.12±0.001	13.75±1.02
OVX	0.89±0.001a	10.02±0.99a
% change	(-20.5%)	(-27.1%)
OVX + red clover	1.00±0.003ab	13.84±0.75b
% change	(-10.7%)	(0.65%)
OVX + Royal jelly	0.96±0.002ab	12.12±0.62
% change	(-14.2%)	(-11.7%)

- Data are mean ± SE of 8 rats.

- (a) Significant different from SHAM group at $p < 0.05$.

- (b) Significant different from OVX group at $p < 0.05$.

- (%) percentage change from SHAM group.

- Abbreviation: SHAM=sham operated group; OVX=ovariectomized; OVX + red clover= OVX group treated orally with red clover (750mg/kg/day) and OVA + Royal jelly= OVX group treated orally with royal jelly (750mg/kg/day).

Table 5: Effect of red clover and Royal jelly treatment on Serum total cholesterol, triglyceride and glucose

	Total cholesterol (mg/dL)	Triglyceride (mg/dL)	Glucose (mg/dL)
SHAM	83.1±3.87	112.8±5.43	131.2±4.86
OVX	94.5±2.43a	135.7±3.59a	154.4±4.98a
% change	(13.7%)	(20.3%)	(17.6%)
OVX+red clover	90.4±3.59a	115.3±3.97b	147.8±2.56a
% change	(8.8%)	(2.2%)	(12.6%)
OVX+Royal jelly	94.4±5.33a	117.8±5.76b	136.6±6.13b
% change	(13.6%)	(4.4%)	(4.1%)

- Data are mean ± SE of 8 rats.

- (a) Significant different from SHAM group at $p < 0.05$.

- (b) Significant different from OVX group at $p < 0.05$.

- (%) percentage change from SHAM group.

- Abbreviation: SHAM=sham operated group; OVX=ovariectomized; OVX + red clover= OVX group treated orally with red clover (750mg/kg/day) and OVA + Royal jelly= OVX group treated orally with royal jelly (750mg/kg/day).

DISCUSSION

In the present study, the estrogenic effect of herbal extract (red clover) or worker bee secretion (Royal jelly) on various physiological changes in ovariectomized (OVX) rats as a model for postmenopausal has been evaluated. A marked atrophy of the uterus has been used as evidence of the success of OVX, it was previously known that the uterine weight constitute a typical marker for estrogenic action, since estrogen play a predominant role in inducing uterine weight gain [20, 12]. The ovariectomy caused reduction in estrogen hormone which accordingly caused a reduction in uterine estrogen receptors. This may produce decrease in the proliferative layers, luminal epithelium, thin storma and myometrium which in accordingly reduces the weight of the uterus [21]. Phytoestrogen are a diverse class of non-steroidal compounds that have an affinity for estrogenic receptors, for the peroxisome proliferator activated receptor (PPAR) family [22].

Many examples of phytoestrogens including flavonoids, isoflavons, coumestans and lignans have a success effect in acting as estrogen [23]. In the present study, administration of red clover or Royal jelly cause significantly higher uterine weight compared to OVX rats. These results are in agreement with Emrah and Suzan [7], who proved the potential estrogenic effect of *Trifolium partinase* (red clover) on uterine estrogen receptors, causing increase in uterine weight, glands and endometrial height. In addition, Kazu *et al.* [9] reported that Royal jelly secreted by worker honey bee has a week estrogenic activity mediated by interaction with estrogen receptors that lead to changes in gene expression and uterine cell proliferation and this also explain the increase in the uterine weight due to Royal jelly treatment in OVX rats in present study.

Modern researches showed that the decreased ovary function of estrogen secretion, and disturbances of hypothalamus-pituitary-ovarian axis cause postmenopausal disorders leading to series of symptoms. Such disruption can have a variety of causes lead to abnormality in hormone secretion. As the main hormone secreted from the ovary, estrogen plays an important role in gonads developments through paracrine/outocrine path ways [24]. This hormone has a wide spread distribution of their receptors throughout the brain and the reduction in estrogen content cause disturbances in these receptors and may lead to neurodegeneration or neuronal loss [25].

Due to OVX, the decrease in estrogen leads to impairment of neuromediator system, through changes in the synthesis, release, reuptake or catabolism of neurotransmitters [26]. King *et al.* [27], recorded an accumulation of 5-hydroxytryptophane (5-HTP) and L-dihydrophenyle alanine (L-DOBA) which induce inhibition in (5-HT) and other catecholamine (DA and NE) synthesis in hypothalamus of OVX rats. This may be explain the decrease in 5-HT and DA content

in OVX rats as compared to their content in sham-operation group in the present study. The estrogen acts on gene expression in serotonin neurons in a manner that could increase 5-HT neurotransmission. That is estrogen treatment to OVX rats cause increase in tryptophan hydroxylase gene [28].

The present study showed that the treatment of OVX rats with red clover or Royal jelly significantly elevated the 5-HT and DA content in the hypothalamus. These results are in similar to those obtained by Shively et al. [29], they reported that the treatment with estrogen replacement extracts increase the 5-HT synthesis and neuronal firing in vitro, and also in vivo by [30].

Many types of phytoestrogen [31] as well as Royal jelly [32] are known to be antioxidant by suppressing formation of reactive oxygen species (ROS) and preventing release of cytochrome C from mitochondria. As previously known that, OVX are associated with increase in free radicles [33], these free radicles are responsible for causing physiological damage to many different organs including the brain. Moreover, neuroprotective effects have been reported for Quercetin (a phytoestrogen) which are probably not mediated via ERs but are rather based on their antioxidant and free radicle scavenging properties [34]. The estrogenic activity of components of red clover and royal jelly may also indirectly contribute to the observed amelioration in 5-HT and DA content up on binding ERs, estrogen up regulates expression of antioxidant enzyme via intercellular signaling pathways. Recently, ERs have been identified in mitochondria [35], and estrogen supplement via phytoestrogen has been reported to increase the level of ERα in mitochondria and to modulate mitochondrial function, resulting in greater energy-producing capacity and decrease ROS production [36].

The neuron that produce gonadotropin releasing hormone (GnRH) are mainly found in the hypothalamus and constitute common final pathway to control LH and FSH and these secretions are controlled by neurotransmitters (5-HT, DA and NE) [37]. There was a great deal of attention in the role of 5-HT in GnRH release [38]. The results of the present study demonstrated that, OVX cause a significant elevation in serum LH and FSH. However, the oral administration of the extract of red clover and the Royal jelly promotes estrogen release and diminishing the ascending FSH and LH in OVX rats. It could be inferred that red clover and royal jelly played a protective role against deleterious changes in aspects of postmenopausal associated hypothalamo-pituitary-ovarian hormones and thus, shifting the endocrine dynamic towards a more physiologically favorable balance.

The role of estrogen in the regulation of glucose homeostasis has been shown previously, for example, postmenopausal women are at risk for increased incidence of obesity, type-2 diabetes, cardiovascular disease and insulin resistant syndrome [39, 40, 41], whereas estrogen therapy reduces the incidence of insulin resistance and type-2 diabetes risks [42], this explain our present results in accumulation of energy stores by elevation of total cholesterol , triglycerides and glucose levels in serum of OVX rats.

The treatment with phytoestrogen (red clover extract) and honey bee secretion (Royal jelly) in OVX rats caused a significant decrease in serum triglyceride in spite of being have no changing in the content of total cholesterol and glucose. These results are in agreement with those obtained by Lee et al. [43] who recorded a decrease in triglyceride levels after treatment with soy protein due to its estrogen constituents and its effects on ERs. It is well known that, cholesterol is synthesized in the liver [44], and the liver is primarily ERα-receptive and therefore the cholesterol lowering effect of estrogen on the ERα in the liver [45]. As previously mentioned that, most of phytoestrogen [9] as well as Royal jelly [46] appear more efficient to ERβ than ERα which make the treatment with these compounds not attached to all ERs and this may explain our present results.

Conclusion: taken together, the results outlined provided strong support for the original hypothesis that red clover and Royal jelly could improve hypothalamic-pituitary-ovarian axis function and normalize the neuroendocrine status, including positive modulating the dopaminergic and serotonergic function and altering postmenopausal –associated pituitary- ovarian hormones changes in a beneficial manner. Furthermore, the red clover and Royal jelly have a potential for further development as safe and effective alternative/ complementary to conventional medication in treating postmenopausal-related syndrome.

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