EFFECTS OF ROYAL JELLY ON BIOCHEMICAL AND REPRODUCTIVE PARAMETERS IN HYPERANDROGENISED FEMALE RATS

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by

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LIST OF ABBREVIATIONS

ANOVA	One-way analysis of variance
10-HDA	Trans-10-hydroxy-2-decenoic acid
CAT	Catalase
DPPH	1,1-diphenyl-2-picrylhydrazyl
E_2	Oestradiol
ELISA	Enzyme-linked immunosorbent assay
FSH	Follicle stimulating hormone
GCMS	Gas chromatography mass spectrometry
GnRH	Gonadotropin-releasing hormone
GPx	Glutathione peroxidase
HDAA	10-hydroxydecanoic acid
LCMS	Liquid chromatography mass spectrometry
LH	Luteinising hormone
MDA	Malondialdehyde
NADPH	Nicotinamide adenine dinucleotide phosphate
OS	Oxidative stress
PCOS	Polycystic ovary syndrome
RJ	Royal jelly
RT	Retention time
s.c	Subcutaneous
S.E.M	Standard error mean
SOD	Superoxide dismutase
T	Testosterone
TAC	Total antioxidant capacity

TBA	Thiobarbituric acid
USA	United States of America
MCF-7	Michigan Cancer Foundation-7
DNA	Deoxyribonucleic acid
mRNA	Messenger RNA

KESAN JELI RAJA KE ATAS PARAMETER BIOKIMIA DAN PEMBIAKAN DALAM TIKUS BETINA YANG HIPERANDROGENIK

ABSTRAK

Sindrom ovari polisistik (PCOS) adalah keabnormalan endokrin yang biasa dihadapi wanita dengan ciri hiperandrogenism, gangguan ovulasi dan ovari polisistik. Jeli raja (RJ) digunakan secara tradisional untuk meningkatkan fungsi pembiakan wanita dan kesuburan. Tujuan kajian ini adalah untuk menganalisis komposisi RJ dan menilai kesan RJ terhadap parameter biokimia dan pembiakan dalam tikus betina yang hiperandrogenik. Empat puluh ekor tikus betina yang belum matang (berumur tiga minggu, 40-50 g) dibahagikan secara rawak kepada 5 kumpulan iaitu Kawalan, Testosteron (T), T + 100RJ (100 mg/kg/hari RJ), T + 200RJ (200 mg/hari RJ) dan T + 400RJ (400 mg/kg/hari RJ). Status hiperandrogenik diaruh dengan memberi testosteron propionate (10 mg/kg) ke dalam peritonium selama tiga minggu diikuti oleh RJ secara oral setiap hari selama empat minggu. Calitan vagina dilakukan setiap hari untuk penilaian kitaran estrus. Di akhir kajian, semua tikus dibunuh dan darah dikumpulkan untuk menilai tahap hormon pembiakan dan penilaian status oksidan-antioksidan. RJ mengandungi sebatian nutrien dan fitokimia dengan fungsi biologi yang bermanfaat termasuk sifat estrogenik dan antioksidan. Berat badan dan paras glukosa di dalam darah ketika berpuasa menunjukkan tiada perbezaan yang signifikan di dalam semua kumpulan. Peratusan kitaran estrus lebih rendah secara signifikan dalam kumpulan T berbanding kumpulan kawalan dan kumpulan T + 200RJ. Aras hormon T dan estradiol (E_2) lebih tinggi secara signifikan manakala hormon perangsang folikel (FSH) kurang signifikan dalam kumpulan T berbanding dengan kumpulan kawalan. Kumpulan T + 200RJ secara signifikannya

meningkatkan aras FSH, manakala aras hormon luteinising (LH), T, dan E_2 berkurang secara signifikan berbanding dengan kumpulan T. Hasil histologi menunjukkan jumlah folikel primer dan sistik adalah lebih signifikan manakala bilangan folikel sekunder dan corpora luteum kurang signifikan dalam kumpulan T berbanding kumpulan kawalan. Di samping itu, kumpulan T + 200RJ secara signifikannya mengurangkan bilangan folikel primer dan sistik dan meningkatkan bilangan folikel sekunder dan corpora luteum berbanding kumpulan T, T + 100RJ dan T + 400RJ tetapi tiada perbezaan dengan kumpulan kawalan. Tahap malondialdehyde (MDA) dan glutathione peroxidase (GPx) secara signifikan lebih tinggi manakala tahap total antioxidant capacity (TAC) adalah kurang signifikan dalam kumpulan T berbanding dengan kumpulan kawalan. Walau bagaimanapun, kumpulan T + 200RJ secara signifikannya menurunkan tahap MDA dan aktiviti GPx, dan meningkatkan tahap TAC berbanding dengan kumpulan T. Penemuan ini menunjukkan bahawa RJ pada dos 200 mg/kg/hari selama 4 minggu meningkatkan tahap hormon pembiakan, kestabilan kitaran estrus, histologi ovari dan status oxidan-antioksidan ovari dalam tikus belum matang yang hiperandrogenik sebagai model tikus PCOS. Perubahan ini boleh dikaitkan sebahagiannya dengan kesan kombinasi dari sebatian fitokimianya yang mempunyai sifat estrogenik dan antioksidan. Kesimpulannya, RJ pada dos 200 mg/kg/hari secara signifikan meningkatkan perubahan biokimia dan pembiakan dalam tikus betina yang hiperandrogenik dan memerlukan kajian lanjut untuk menilai mekanisme tindakan yang tepat.

EFFECTS OF ROYAL JELLY ON BIOCHEMICAL AND REPRODUCTIVE PARAMETERS IN HYPERANDROGENISED FEMALE RATS

ABSTRACT

Polycystic ovary syndrome (PCOS) is a common endocrine abnormality in women characterised by hyperandrogenism, ovulatory dysfunction and polycystic ovary. Royal jelly (RJ) is traditionally consumed for enhancement of female reproductive function and fertility. The aims of this study were to analyse the composition of RJ and to evaluate the effects of RJ on biochemical and reproductive parameters in hyperandrogenised female rats. Forty immature female rats (three weeks old, 40-50 g) were randomly divided into 5 groups i.e. Control, Testosterone (T), T+100RJ (100 mg/kg/day RJ), T+200RJ (200 mg/kg/day RJ) and T+400RJ (400 mg/kg/day RJ) groups. Hyperandrogenic state was induced by giving testosterone propionate (10 mg/kg) intraperitoneally for three weeks followed by RJ via oral gavage daily for four weeks. Vaginal smear was done daily for assessment of oestrus cycle. At the end of the study, all rats were sacrificed and blood was collected for reproductive hormones levels and oxidant-antioxidant status assessment. RJ contained nutrient and phytochemical compounds with beneficial biological activity including oestrogenic and antioxidant properties. Body weight gain and fasting blood glucose level showed no significance differences among all groups. The percentage of regular oestrus cycle was significantly lower in T group compared to control and T+200RJ groups. T and oestradiol ($E_{2)}$ levels were significantly higher while follicle-stimulating hormone (FSH) was significantly lower in T group compared to control group. In T+200RJ group, FSH level was significantly higher while luteinizing hormone (LH), T and E_2 levels were significantly lower compared to T group. The

histological results showed the numbers of primary and cystic follicles were significantly higher while numbers of secondary follicle and corpora luteum were significantly lower in T group compared to control group. In addition, the numbers of primary and cystic follicles were significantly lower and numbers of secondary follicle and corpora luteum were significantly higher in T+200RJ compared to T, T+100RJ and T+400RJ groups but not significantly different from control group. Malondialdehyde (MDA) and glutathione peroxidase (GPx) activities were significantly higher while total antioxidant capacity (TAC) level was significantly lower in T group compared to control group. However, in T+200RJ group, MDA level and GPx activity were significantly lower while TAC level was significantly higher compared to T group. These findings might suggest that RJ at 200 mg/kg/day for 4 weeks improves reproductive hormone levels, oestrus cycle regularity, ovarian histology and ovarian oxidant-antioxidant status in hyperandrogenised immature rats as PCOS rat model. This effect could be attributed partly to the combined effects of its phytochemical compounds that have oestrogenic and antioxidant properties. In conclusion, RJ at the dose of 200 mg/kg/day significantly improves biochemical and reproductive changes in hyperandrogenised female rats which need further study to evaluate its exact mechanism of action.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Hyperandrogenism is one of the most important characteristics of polycystic ovary syndrome (PCOS). The exact cause of PCOS is unknown, but it was known that PCOS may be developed by exposure to surplus androgens during the reproductive years. Animal studies have shown fetus that exposed to excess androgens produces symptoms of PCOS during adult (Sullivan *et al.*, 2004; Abbott *et al.*, 2008). Excess androgen activity can modify gonadotropin-induced progesterone and oestrogen synthesis in the follicles (Wachs *et al.*, 2008). Androstenedione and testosterone (T) are aromatised by P450 aromatase into estrone and estradiol. However, when the activity of P450 aromatase reduces, it will cause increase in production of ovarian androgen which then develop into the PCOS (Chen *et al.*, 2015).

The European Society for Human Reproduction and the American Society of Reproductive Medicine Rotterdam Consensus Meeting (2003) has proposed the presence of at least two of the following symptoms; oligo-/or anovulation, hyperandrogenism, and ultrasound polycystic ovaries, are sufficient to confirm the diagnosis of PCOS (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus, 2004). Patients with PCOS are known to have incidence of oxidative stress (OS) and insulin resistance which may influence female reproductive system (Kandasamy *et al.*, 2010).

As OS is well-defined as an imbalance between the response of reactive oxygen species and antioxidant defence system that can produce the oxidative damage (Agarwal *et al.*, 2012). OS in cells can be protected by enzymatic antioxidant such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and by non-enzymatic antioxidants, such as glutathione or antioxidant vitamins (vitamins A, C, E) (Aten *et al.*, 1992). It has been reported that rats treated with dehydroepiandrosterone have an increased ovarian oxidative stress in which there are diminished both CAT activity and glutathione content, and enhanced lipid peroxidation (Elia *et al.*, 2006). Furthermore, decreased total antioxidant status and increased level of protein carbonyl have been found in serum of PCOS patients suggesting the presence of OS in PCOS (Fenkci *et al.*, 2003).

There has been great interest in the role of naturally created agents for PCOS. Natural products, such as *Aloe barbadensis* (Maharjan *et al.*, 2010), spearmint (Grant, 2010), ginseng saponin (Pak *et al.*, 2005) and cinnamon (Wang *et al.*, 2007) are demonstrated to have protective roles to overcome PCOS. *Aloe barbadensis* can alter oestrus cycle, hyperglycemic condition and steroidogenic enzyme activity on letrozole-induced PCOS rat model (Maharjan *et al.*, 2010). Spearmint is able to give a protective effect against PCOS phenotype by reducing testosterone (T) level and increasing luteinising hormone (LH) and follicle stimulating hormone (FSH) levels (Grant, 2010). A pilot study on oral administration for 8 weeks of cinnamon extract enhances insulin sensitivity in non-diabetic PCOS women (Wang *et al.*, 2007). Moreover, Korean red ginseng total saponin is found to partly inverse the abundance of nerve growth factor expression in the estradiol-valerate-induced PCOS rat model (Pak *et al.*, 2005).

Recently, royal jelly (RJ) has established particular attention as numerous studies reported RJ to possess high antioxidant property as well as other bioactivity properties (Salazar-Olivo & Paz-González, 2005; Silici *et al.*, 2009; Ramadan & Al-Ghamdi, 2012). For many years, the beneficial effects of RJ on fertility and sexual ability have received much attention from the researchers. RJ or bee's milk is yellowish-white acidic secretion produced by the hypopharyngeal glands of the worker honeybees with a slightly pungent odour and taste (Ramadan & Al-Ghamdi, 2012). For the first 3 days, RJ is the only food consumed by young larvae for their development process, while it also the specific food consumed by the queen bee throughout its life exclusively (Isidorov *et al*., 2009). The queen bee which is exclusively fed with RJ, is characterised by longer lifespan and well-developed gonads compared to worker bees (Popescu *et al.*, 2008; Morita *et al.*, 2012).

The RJ is considered as a vital product among honeybees production with high biological and nutritional properties. Generally, RJ consists of high nutritional values due to abundant amounts of water (60 – 70 %), proteins (9 – 18 %), sugars (7–18 %), lipids (3–8 %), minerals, vitamins and essential amino acids. While, lyophilized RJ consists of less than 5 % water, 27–41 % proteins, 15–30 % lipids and 22–31 % carbohydrates (Sabatini *et al.*, 2009; Bogdanov, 2015). Despite abundant studies have been done to detect the chemical composition of RJ, the knowledge in this field has continued incomplete. RJ possesses some biological properties such as anti-inflammatory (Yanagita *et al*., 2011), anti-cancer (Nikokar *et al*., 2013) and immunomodulatory properties (Gasic *et al*., 2007). RJ also has been reported to have other properties including antioxidative activity (Liu *et al*., 2008; Jamnik *et al*., 2007), increased fertility (Elnagar, 2010), antidepressant effects (Ito *et*

al., 2012), antiproliferative effects (Nakaya *et al*., 2007), oestrogenic activity (Mishima *et al.*, 2005; Suzuki *et al.*, 2008), prevents osteoporosis (Yanagita *et al*., 2011), reduces oxidation of intracellular cell (Jamnik *et al.*, 2007), ameliorates insulin resistance (Zamami *et al*., 2008) and protect liver damage (Kanbur *et al*., 2009).

Studies revealed RJ to have oestrogenic activity similar to the other exogenous steroid hormones (Hidaka *et al.*, 2006; Nakaya *et al.*, 2007) and its fatty acids are detected to mimic human oestrogen (Suzuki *et al.*, 2008). In other study, RJ increase plasma T levels, sperm maturation, sperm motility and sperm count (Zahmatkesh *et al.*, 2014). Intramuscular injection of RJ at 400 mg/day in conjunction with a 12-day intravaginal progesterone treatment significantly improves pregnancy rates and oestrus response in ewes (Husein & Kridli, 2002). Oral administration of RJ (200, 400 and 800 mg/kg) to heat stressed male rabbits improves seminal parameters such as improves seminal fructose, seminal ejaculated volume, sperm motility and total sperm output, increases level of T, reduces abnormal and dead sperm concentrations as well as improves liver and kidney functions during summer infertility (Elnagar, 2010).

1.2 Justification of the study

Previous studies show that RJ possesses many health benefits. However, RJ is not quite well studied with sufficient scientific data compared to other bee products such as honey, propolis, bee bread and bee pollen. To date, no study has been stated on the possible beneficial outcome of RJ on biochemical and reproductive parameters in human and animal models of PCOS. This study may provide

preliminary findings for further clinical research on the beneficial effect of RJ among PCOS patients. Perhaps these findings can help in promoting the use of local RJ in complementary medicine and may help the local beekeepers in marketing their products and subsequently may boost Malaysian agriculture industry.

1.3 The hypotheses of the study

1) RJ has nutrients and beneficial phytochemical compounds.
2) RJ significantly improves body weight gain and fasting blood glucose in hyperandrogenised female rats.
3) RJ significantly improves oestrous cycle, relative ovarian weight and ovarian histology (number of primary follicle, secondary follicle, corpus luteum and cystic follicle) in hyperandrogenised female rats.
4) RJ significantly improves reproductive hormones levels (T, E_2, LH and FSH) in hyperandrogenised female rats.
5) RJ significantly improves ovarian oxidant-antioxidant status (MDA, TAC, SOD, GPx and CAT) in hyperandrogenised female rats.

1.4 Objectives of study

1.4.1 General objective

To determine the effects of RJ of honeybee (*Apis mellifera*) on biochemical and reproductive parameters in hyperandrogenised female rat as animal model of PCOS.

1.4.2 Specific objectives

1) To determine and compare the composition of two RJ samples from Malaysia.
2) To determine the effects of RJ on bodyweight gain and fasting blood glucose in hyperandrogenised female rats.
3) To determine the effects of RJ on relative ovarian weight, oestrus cycle and ovarian histology (number of primary follicle, secondary follicle, corpus luteum and cystic follicle) in hyperandrogenised female rats.
4) To determine the effects RJ on reproductive hormones levels (T, E_2, LH and FSH) in hyperandrogenised female rats.
5) To determine the effects RJ on ovarian oxidant-antioxidant status [malondialdehyde (MDA), total antioxidant capacity (TAC), superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT)] in hyperandrogenised female rats.

CHAPTER 2

LITERATURE REVIEW

2.1 Polycystic ovary syndrome

2.1.1 Definition and prevalence

The PCOS is defined as a common endocrine disorder with a set of symptoms that exhibiting a wide spectrum of clinical manifestations during their reproductive years (Franks, 2002). PCOS was attempted to be defined early during a conference by National Institutes of Health in 1990, followed by Rotterdam conference which sponsored by the European Society for Human Reproduction and Embryology and the American Society for Reproductive Medicine. Then, during 2006, Androgen Excess and PCOS Society presented a new diagnostic criterion.

Prevalence of PCOS estimation can be arranged depends on the diagnostic criteria. By using the National Institutes of Health 1990 criteria, about 6-8 % women in the world are having PCOS. However larger figure is obtained by using Rotterdam criteria (March *et al.*, 2010). About 4 % of women in United State and 6.5 % in Spain were affected by PCOS during their reproductive ages (Knochenhauer *et al.*, 1998; Asuncion *et al.*, 2000). Table 2.1 shows the international standard of diagnostic criteria according to National Institutes of Health, Rotterdam conference and Androgen Excess and PCOS Society (National Institutes of Health, 2012).

Table 2.1: Standard diagnostic criteria of polycystic ovary syndrome.

Definition	Diagnostic criteria
National Institutes of Health, 1990.	Defined by the presence of: 1) Hyperandrogenism and 2) Ovulatory dysfunction (anovulation, oligoovulation, oligomenorrhea and amenorrhea)
Rotterdam conference, 2003.	Defined by the presence of: 1) Hyperandrogenism and 2) Ovulatory dysfunction 3) Ovarian morphology (Twelve or more 2-9 mm follicles and/or at least one enlarged ovary (>10 mm)
Androgen Excess and PCOS Society, 2006.	Defined by the presence of: 1) Hyperandrogenism and 2) Ovarian dysfunction (ovulatory dysfunction or ovarian morphology).

PCOS: polycystic ovary syndrome.

In 1935, PCOS in many cases of women was identified by Stein and Leventhal characterised by hirsutism, presence of polycystic in the ovary and irregular menstruation (Stein & Leventhal, 1935). However, its pathogenesis remains unknown. Gradually, more studies and information are assembled by time with more arranged symptoms, cause and related condition, diagnostic procedures and variety options to treat PCOS (Sheehan, 2004). However, the causes of this disease remain unknown with too many theories. Yet, some testaments and studies show that genetic factor and family history cause the affected ladies with the existence symptoms of PCOS before their menarche (Franks *et al.*, 2006).

2.1.2 Pathogenesis of polycystic ovary syndrome

PCOS is a complicated disease predisposed by the synergistic effect of environmental aspects, genetic, along with hormonal and metabolic processes. In ovarian tissue of PCOS women, the theca cell in the ovary synthesises androgen due to stimulation of LH. Androgen biosynthesis is facilitated by enzyme cytochrome P-450c17, with collaboration of 17a-hydroxylase and 17, 20-lyase activities to form androstenedione. Afterwards, the androgenic steroid is transformed by 17b-hydroxysteroid dehydrogenase to form T. This androgenic steroid also can be aromatised to form estrone by cytochrome P-450arom; the aromatase enzyme (Ehrmann, 2005). Ovarian theca cells in PCOS patients are more effective in translating androgenic precursors to T than the theca cells of normal woman (Nelson *et al.*, 1999; Nelson *et al.*, 2001). It is well known that FSH regulates the aromatase activity of granulosa cells while LH regulates the androgenic synthesis of theca cells.

As LH level increases relative to that of FSH concentration, the ovaries synthesise more androgen and cause hyperandrogenism.

(i) Hyperandrogenism

Hyperandrogenism or androgen excess is a common endocrine disorder affecting reproductive-age women with complex diagnostic challenge to be handled by the practicing physicians and the clinical investigators (Yildiz, 2006). Hirsutism is commonly become the sign of hyperandrogenism clinically with the excess thick body hair on the face, abdomen, chest or upper thighs which starts after menarche as well as arisen of acne. Androgenic alopecia is male pattern hair loss while virilisation symptoms such as muscle bulk in women and deepening of the voice as a result of excess androgen production. Diagnosis of hyperandrogenism is usually accompanied by hirsutism, androgenic alopecia, acne and virilisation (Diamanti-Kandarakis, 2009). The stimulus of hypothalamic gonadotropin-releasing hormone (GnRH) regulates the relative proportion of LH and FSH hormones that will be synthesised within the gonadotrope. Elevated pulse frequency of hypothalamic GnRH in PCOS will encourage transcription of the β-subunit of LH over the β-subunit of FSH. The changes in gonadotropins will cause deficient development in the follicles resulting anovulation and irregular menstruation. This arrested follicle will form a cystic structure as a sign of PCOS (Oakley *et al.*, 2011).

(ii) Insulin resistance

Insulin resistance can negatively affect regularity of pituitary hormones and follicular development. Insulin resistance is a condition where the cells do not respond to the effects of insulin causing elevated glucose level in the blood (Diamanti-Kandarakis & Dunaif, 2012). Patients at the age of 40 have developed

glucose intolerance which is impaired by type 2 diabetes mellitus (Legro *et al.*, 1999). Insulin resistance is associated with acanthosis nigricans resulting from insulin's action via insulin-like growth factor receptors which triggers the excess growth of keratinocyte that produces velvety skin patches (Rotstein, 2013). Insulin involves directly and indirectly on hyperandrogenism and PCOS and cause hyperinsulinemia. Hyperinsulinemia is defined as an excessive insulin secretion in blood that resulting from insulin resistance (Diamanti-Kandarakis, 2006). It plays a role in increasing ovarian T production and cause anovulation (Loverro *et al.*, 2001). Insulin together with LH will boost production of androgen in theca cells. Insulin also inhibits hepatic synthesis of sex hormone–binding globulin, the key circulating protein that binds to T. This will increase T circulation. Hyperinsulinemia will cause a rise in free T concentration when total T concentration is also increased (Ehrmann, 2005). Decreased level of insulin in PCOS patients by consumption of metformin could reduce free T level and improve ovulatory function (Nestler *et al.*, 1998).

PCOS involves ovarian structure development that starts at an early age in life probably during ovarian development stage and oogenesis (Franks, 2002) as well as by genetic factors. This is confirmed by a variety of studies on young girls that display the symptoms of PCOS before their first menstruation. PCOS also occurs among their siblings and relatives (Franks, 2008). More importantly, exposure to androgen during early life in female either during prenatal, perinatal and postnatal periods can cause the presence of PCOS characteristics in adulthood (Wu *et al.*, 2010). Thus, early androgen exposure during adrenarche has association with development of PCOS in future. Moreover, adrenarche girls with irregular menstrual cycle have higher potential to have elevated androgen levels compared to normal

subjects with regular menstrual cycle (Pinola *et al.*, 2012). Previous studies on human and animal also have proposed that early exposure to androgen might contribute to arise in PCOS (Abbott *et al.*, 2009; Ibanez *et al.*, 2011). Hence, androgen treatment before adulthood in animal studies perhaps will closely resemble PCOS in human.

(iii) Genetic factors

CYP19 aromatase gene is suggested to contribute to the excess androgen in PCOS patients (Belgorosky *et al.*, 2003). PCOS cases appear to be inherited in families. About 40 % of sisters from PCOS women and 35 % of premenopausal women have a predictable role of genetics in PCOS (Kahsar-Miller *et al.*, 2001). PCOS is clustered in families when both symptoms of hyperinsulinaemia and hyperandrogenaemia are detected in siblings (Franks & McCarthy, 2004). Another study assessed by glucose and fasting insulin levels among the family members of PCOS shows that person who has PCOS but with consistent menstruation has less insulin sensitivity than their unaffected sisters (Legro *et al.*, 2002).

(iv) Oxidative stress

OS is defined as imbalance of antioxidant defences with reactive oxygen species (free radicals) (Burton & Jauniaux, 2011). Elevated reactive oxygen species in the body can cause impairment of cell, protein and lipid. Reactive oxygen species are consist of free radical oxygenated molecules, like superoxide, hydroxyl radical, hydrogen peroxide, singlet oxygen that have unpaired electron in the external orbit and could survive independently (Zuo *et al.*, 2016). OS has an association with pathogenesis and complication of PCOS when reactive oxygen species accumulate

faster than antioxidants defence systems. Oxidation circumstances can cause inter- and/or intramolecular cross-linking which can lead to enzyme inactivation and protein degradation. During the ovulatory process, follicles can produce reactive oxygen species which trigger OS and can promote to poor oocyte quality (Govindarajan *et al.*, 2015).

Antioxidants are the compounds that are able to detoxify excess reactive oxygen species to prevent oxidative damage (Agarwal *et al.*, 2012). Oxidative damage in biomolecules like protein, lipids, carbohydrate and nucleic acids can harm the cell and cause cell death. Furthermore, the presence of antioxidant can prevent the damage by reactive oxygen species and has been reported to play a role in infertility and generally in the female reproductive system (Kuşçu & Var, 2009). Antioxidants such as peroxidase, CAT, SOD and low molecular weight molecules such as tocopherol, ascorbic acid and polyphenols play important roles in protecting the body from oxidative damage (Nagai *et al.*, 2001).

Many investigations on antioxidant markers in PCOS patients are promising to correlate OS with PCOS circumstances and metabolic syndrome manifestations. Previous study has found that PCOS patients show the presence of OS compared to normal women as they have significantly higher level of SOD and MDA activities but lower activity of GPx (Murri *et al.*, 2013). In another clinical study, PCOS patients have significantly increased MDA, a lipid peroxidation marker as well as decreased CAT activity and decreased glutathione, vitamins C and E levels suggesting of higher free radicals that lead to OS in PCOS patients (Al-Azzawie & Hameed, 2010). Similarly, PCOS patients also have significantly increased MDA,

GPx and homeostasis model assessment for insulin resistance levels with concomitant reduction in total antioxidant status and glutathione levels and CAT activity (Kandasamy *et al.,* 2010). *In vivo* study on PCOS induced rats by estradiol-valerate shows that TAC and SOD levels and CAT activities were significantly decreased but MDA level is significantly increased in PCOS treated groups suggesting the presence of OS which may cause PCOS (Jafarisani *et al.*, 2016). The suggested pathogenesis of PCOS is summarised in Figure 2.1.

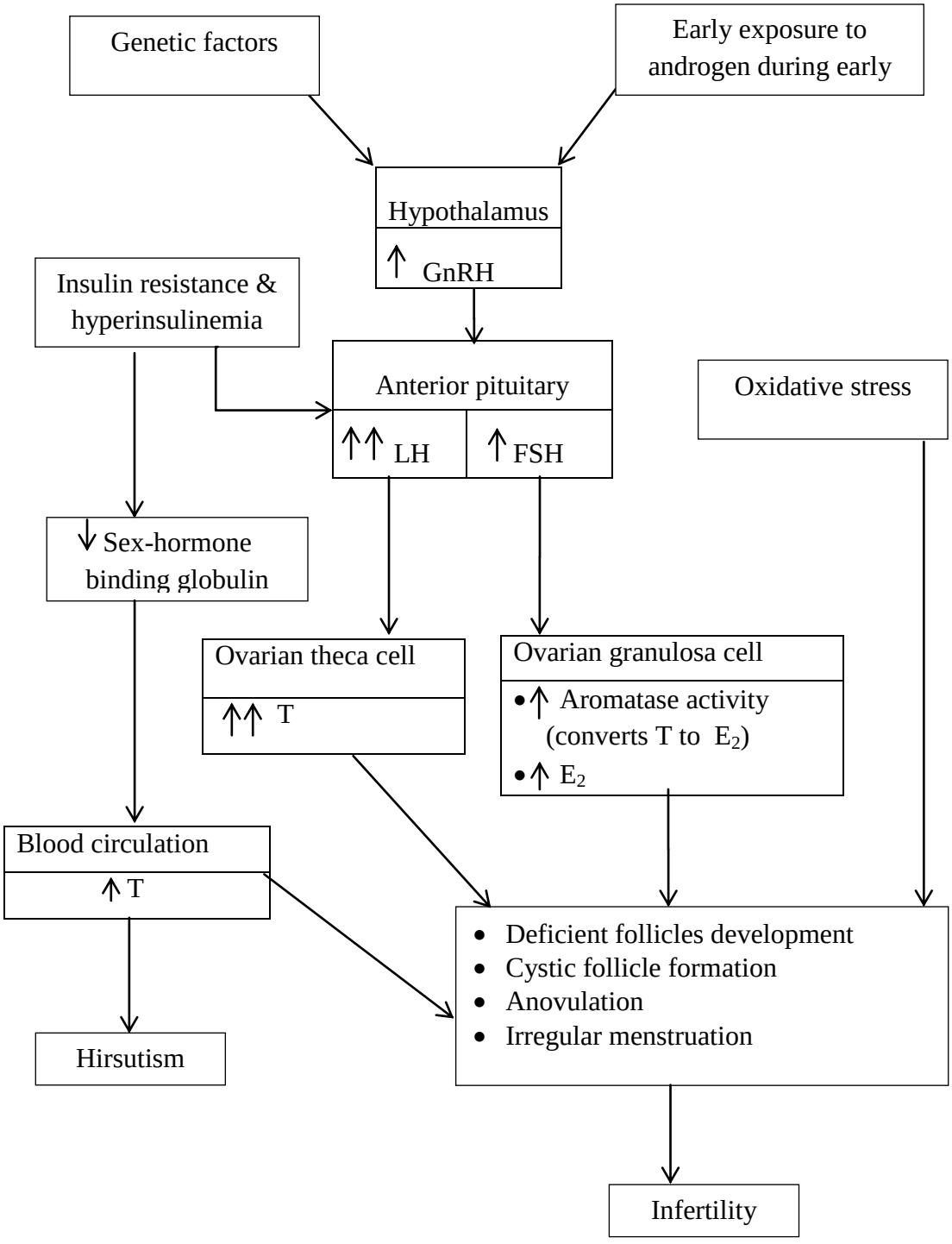

Figure 2.1:Pathogenesis of polycystic ovary syndrome
GnRH: gonadotropin-releasing hormone, T: testosterone, $E_{2:}$ oestradiol,
LH:leutinising hormone, FSH: follicle-stimulating hormone,

↑: high level, ↑↑: very high level.

2.1.3 Clinical features of polycystic ovary syndrome

Even though PCOS is an endocrine disease, it can affect metabolic, reproductive and psychological disorders (Garad *et al.*, 2011). Clinical features of PCOS are listed in Table 2.2 (Teede *et al.*, 2010).

Table 2.2: Clinical features of polycystic ovary syndrome

Feature	Criteria
(a) Reproductive features.	Hyperandrogenism, ovulatory and menstrual dysfunction, hirsutism, infertility, miscarriage, pregnancy-induced diabetes (gestational diabetes), pregnancy-induced hypertensive disorders, complications in pregnancy, neonatal complications and increased endometrial hyperplasia.
(b) Metabolic features.	Metabolic syndrome, type 2 diabetes, dyslipidaemia, insulin resistance, high rates of premature impaired glucose tolerance and increased cardiovascular risk factors.
(c) Psychological features.	Depression, anxiety, reduced quality of life, poor self-esteem and negative body image.

2.1.4 Animal models for polycystic ovary syndrome

There is rapidly growing literature on PCOS, which needs studies at many levels including animal study. To investigate this complex disease, appropriate animal models must be applied. Many years ago, researchers have identified numerous animal models to imitate pathophysiological changes that closely resemble PCOS condition which can contribute to valuable information on PCOS.

Rodents are the most common animal model used to study PCOS because of its small size, easy to be handled, high reproduction index, short life- span and have different genetic strains (Shi *et al.*, 2012). Research on animal models also becomes more favourable due to their similarities in key steps in reproduction of mammalian. A considerable amount of literature has been published on adult and immature rats. Many hyperandrogenised animal models have been developed to identify the aetiology of PCOS (Abbott *et al.*, 2005; Oakley *et al.*, 2011; Yaba & Demir, 2012; Dikmen *et al.*, 2012). A study has provided ample support to claim the effects of androgen administration to immature rats that contributed to PCOS morphology for short-term treatment and likewise follicle atresia is elevated for medium-term treatment (Honnma *et al.*, 2006).

A recent animal study has been done on immature rats induced by dehydroepiandrosterone show the ovaries are steroidogenically more active than those of controls, suggested that the dehydroepiandrosterone acts on the ovary directly and due to the pituitary- hypothalamus axis action. While this model resulted in changes in hormone levels and presence of ovarian cystic and lead to acyclic and

ovulatory (Kavitha *et al.*, 2016). Pre-pubertal androgen animal models have been developed to increase the level of androgen during prepubescent of the animal. Current research appears to validate the condition whereby a study with T propionate induction in 3 week old immature female rats has led to insulin resistance, anovulation, polycystic ovaries and impaired progesterone production (Beloosesky *et al.*, 2004). Therefore, administration of exogenous androgens in immature rats has resulted in permanent damage to ovarian and metabolic features of PCOS (Manneras *et al.*, 2007).

2.1.5 Treatment for polycystic ovary syndrome

PCOS is a distressing disorder for women with complicated diagnosis and management for healthcare professionals thus become scientific challenge for researchers. In recent years, there is public interest in using natural products instead of advancement of modern medicine. People become revert to natural products which can satisfy them and when their illness could not be cured by conventional methods (Pal & Shukla, 2003). Natural products receive growing attention as more people become concern of their health. Thus, many natural products have been discovered as complementary therapies to treat PCOS. One of the most vital properties of the natural products is its antioxidant property that possibly will contribute to the treatment and prevention of illnesses.

Recent study in the field of treating PCOS patients has led to a renewed interest in using Epimedium herb (*Herba epimedii*). It reduces T level by its anti-androgen property and it is suggested that it can treat hirsutism in PCOS (Grant, 2010). Flax seed is reported to significantly improved menstrual cycles, lower

ovarian weight and number of follicles in polycystic ovaries, and do not alter the blood glucose, body weight and hirsutism (Fatima *et al.*, 2015). In addition, *Tephsia purpurea* significantly improves the oestrus cycle in PCOS induced rats (Jitendra & Pravin, 2012). Similarly, a study on T propionate-induced PCOS rats treated with *Pergularia daemia* also shows normalising of oestrus cycle to 80-90% normal level (Bhuvaneshwari *et al.*, 2015). Moreover, treatment of letrozole- induced PCOS rat model with *Mimosa pudica Linn.* significantly controls hyperandrogenism by lowering the damage in ovary histology and endrocrinological changes (Mamata *et al.*, 2013). Curcumin treatment in letrozole-induced PCOS female rats, has shown that curcumin gives a comparable effect to Clomiphene citrate as it restores lipid profile, reduces T level, increases progesterone and E_2 levels, reduces glutathione level, SOD and CAT activities and as well as normalise the ovarian morphology (Reddy *et al.*, 2016).

2.2 Royal jelly

2.2.1 Definition and composition of royal jelly

The RJ or bee's milk, is a bee product with creamy yellowish colour secreted by the mandibular glands and hypopharyngeal worker bees (Page & Peng, 2001). It has pungent odour and acidic taste and is enriched with carbohydrates, proteins, vitamins and minerals which encourage growth and fertility of the queen bee. RJ provides nutrition and protection for fast-developing larvae and also for the queen for her whole life (Kolayli *et al.*, 2015). RJ offers huge potential of biological properties with pharmaceutical potentials. It is traditionally used as a supplement and in cosmetic products. However, the composition of RJ can be affected by the duration of harvesting, storage and processing methods, species of honeybee as well as

geographical origin (Isidorov *et al*., 2009; Liming *et al*., 2009). Hence, identifying the composition of RJ is important to assess the freshness, adulteration and quality (Sabatini *et al*., 2009).

Quality and standardisation of RJ has been proposed as follows: water 60-70 %, proteins 9-18 %, carbohydrates 7-18 %, lipids 3-8 %, 10-hydroxy-2-decenoic acid 1.4 %, ash 0.8-3.0 % with pH of 3.4-4.5 and acidity 3.0-6.0 ml 0.1N NaOH/g (Sabatini *et al*., 2009). Lyophilised RJ comprises less than 5 % of water, 27–41 % of proteins, 22–31 % of carbohydrates and 15–30 % of lipids (Popescu, 2008). RJ contains large amount of water to supply the freshness and maintain the moisture in the hive by the natural hygroscopicity of RJ. It is also rich with vitamin B complex including pyridoxine (vitamin B6) and pantothenic acid (vitamin B5) and contains pure acetylcholine (Barnutiu *et al*., 2011).

(i) Proteins

RJ contains of proteins that makes up 50 % of dry mass in RJ composition (Schonleben *et al.*, 2007). These proteins encompass of primarily major RJ proteins with the most abundance is primarily major RJ proteins 1. Royalactin is the most important member of primarily major RJ proteins 1 that presence in RJ. It promotes development of ovary, increases bodyweight and reduces duration of development in honeybees and *Drosophila melanogaster* (Kamakura, 2011). RJ also contains amino acids which composed of highest percentage includes proline, glutamate, aspartate, b-alanine, serine, lysine and phenylalanine (Boselli *et al.*, 2003). From previous study, RJ proteins have biological activities such as immunoregulatory and anti-allergic properties (Simuth & Republic, 2001).

(ii) Carbohydrates

Carbohydrates consist about 30 % from dry matter of RJ. Carbohydrates in RJ mostly consist of glucose, fructose and sucrose (Esta & Sesta, 2006) and some with little amount of maltose, isomaltose, raffinose, trehalose, melezitose, gentiobiose and erlose (Sabatini *et al.*, 2009).

(iii) Lipids

About 3-6 % of lipids can be found in RJ with about 80 % to 90 % are consisted of free fatty acid and the rest are sterols, neutral lipids and hydrocarbon (Kodai *et al.*, 2007). The most vital fatty acid is 10-hydroxy-2-decenoic acid (10-HDA) which can only be found in RJ. Previous study showed that 10-HDA and other fatty acid in RJ exhibit antibacterial activity (Melliou & Chinou, 2005). 10-HDA also has various biological properties such as anti-inflammatory (Yang *et al.*, 2010), immunomodulatory (Gasic *et al.*, 2007; Sugiyama *et al.*, 2012), collagen promoting (Park *et al.*, 2012), antiangiogenic (Izuta *et al.*, 2009) and oestrogenic activities (Suzuki *et al.*, 2008).

(iv) Vitamins

RJ is rich in vitamin A (1.10 mg/100g), B1 (2.06 mg), B2 (2.77 mg), B6 (11.90 mg), B12 (0.15 mg), B5 (52.80 mg), B9 (0.40 mg), C (2.00 mg), D (0.20mg) and E (5.00 mg) (Barnutiu *et al.*, 2011).

(v) Minerals

Minerals in RJ represent about 0.8 to 3 % which are enriched with copper, calcium, iron, lead, aluminium, magnesium, manganese and potassium (Bogdanov, 2015).

(vi) Water

Among all bee products, RJ is the only one with the largest amount of water (Barnutiu *et al.*, 2011). Water content commonly is within the range of 60-70 % (Krell, 1996; Garcia-Amoedo & Almeida-Muradian, 2007). A study done by using large samples of RJ shows that 60-70 % of its water content (Kanelis *et al.*, 2015) which reflect the moisture variation in nature. The constant water content in the hive is probably caused by natural hygroscopicity of RJ in order to maintain the humidity in the hive (Sabatini *et al.*, 2009).

(vii) Other compounds

Small amount of nuclotides such as guanosine, adenosine, uridine, gluconic acid, benzoic acid, malic acid, lactic acid (Isidorov *et al.*, 2009) and acetylcoline (Wei *et al.*, 2009) are found in RJ. These substances are accumulated as volatile organic compounds that have a protective effect against parasites as well as other microorganisms (Isidorov *et al.*, 2009).

2.2.2 Biological activities of royal jelly

Biological activities of RJ are numerous and can be associated with the trace elements itself. However, the physiological effects of RJ are not fully discovered in

human. Yet, many studies have been initiated to continuously investigate some biological effects of RJ.

(i) Antioxidant activity of royal jelly

Antioxidant activity is attributed to fatty acids (Kolayli *et al.*, 2015), proteins and polyphenolic compounds (Nagai *et al.*, 2006; Guo *et al.*, 2009). RJ harvested later than 24 hour after the larval transfer might lower the total polyphenolic contents and lead to a reduction in the antioxidant activities. It was suggested the anti-oxidative level in RJ will be reduced with time, and therefore this activity can be used to determine the quality of RJ (Liu *et al.*, 2008). Some studies have reported on antioxidant property of RJ. A study on diabetic male rats shows that RJ significantly reduces MDA level, DNA damage and chromatin abnormalities, and increases sperm count, testicular weight, motility and viability (Ghanbari *et al.*, 2015). In a clinical study, RJ supplementation significantly improves OS marker (TAC level) and insulin resistance in type 2 diabetic patients (Shidfar *et al.*, 2015).

Interestingly, RJ has the potential to protect DNA tissue against the oxidative damage. The level of 8- hydroxy-2-deoxryguanosine, an oxidative stress marker in kidney DNA and serum of mice after RJ treatment for 16 weeks are significantly reduced and the life span is significantly increased, probably due to the mechanism of reduction in oxidative damage (Inoue *et al.*, 2003).

(ii) Neurotrophic action of royal jelly

RJ is found to have acetylcholine (Wei *et al.*, 2009), a chemical that acts as a neurotrasmitter in nervous system. Thus, many studies have been done to investigate