ผลของเคอร์ซีตินต่อการสร้างอสุจิและสเตอรอยด์ฮอร์โมน ในหนูแรทปกติและหนูแรทที่ถูกตัดอัณฑะ

นางสาวลดาชาติ แต่พงษ์โสรัถ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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EFFECTS OF QUERCETIN ON SPERMATOGENESIS AND STEROIDOGENESIS IN INTACT AND ORCHIDECTOMIZED RATS

Miss. Ladachart Taepongsorat

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy Program in Physiology (Interdisciplinary Program)

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ลดาชาติ แต่พงษ์โสรัถ: ผลของเคอร์ซีตินต่อการสร้างอสุจิและสเตอรอยด์ฮอร์โมนในหนูแรทปกติและหนูแรทที่ถูกตัดอัณฑะ (EFFECTS OF QUERCETIN ON SPERMATOGENESIS AND STEROIDOGENESIS IN INTACT AND ORCHIDECTOMIZED RATS) อ.ที่ปรึกษา: รศ.ดร.ประคอง ตังประพฤทธ์กุล, อ.ที่ปรึกษาร่วม: รศ.ดร.สุจินดา มาลัยวิจิตรนนท์, อ.ดร.นพดล กิตนะ, 136 หน้า

การวิจัยครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของเคอร์ซีตินซึ่งเป็นสารฟลาโวนอยด์ที่พบได้ในพืชหลายชนิดรวมทั้งพืชสมุนไพรของไทยที่ใช้ในการรักษาการทำงานที่ผิดปกติของการสืบพันธุ์ ต่อการสร้างอสุจิและสเตอรอยด์ฮอร์โมนในหนูแรทและหนูที่ถูกตัดอัณฑะออกโดยใช้หนูขาวเพศผู้สายพันธุ์ Sprague-Dawley อายุ 8 สัปดาห์

จากการศึกษาพบว่าเมื่อฉีดเคอร์ซีตินเข้าทางใต้ผิวหนังของหนูแรทปกติ ขนาด 0 (Q0), 30 (Q30), 90 (Q90) หรือ 270 (Q270) มก/กก/วัน เป็นเวลา 3, 7 หรือ14 วัน และเพิ่มเวลาเป็น 28 วันในกลุ่ม Q0 และ Q90 พบว่าการมีผลต่ออวัยวะสืบพันธุ์ (อัณฑะ, ท่อพักเชื้ออสุจิ-ท่อนำเชื้ออสุจิ, ต่อมลูกหมากและถุงน้ำอสุจิ), ฮอร์โมนเพศ (testosterone, LH, FSH) และคุณภาพอสุจิ (การเคลื่อนไหว, การมีชีวิตและจำนวนของอสุจิ) ขึ้นอยู่กับขนาดและระยะเวลาที่หนูได้รับเคอร์ซีติน ระยะเวลาที่สั้นที่สุด (3 วัน) เคอร์ซีตินทุกขนาด ไม่มีผลต่อน้ำหนักและการเปลี่ยนแปลงลักษณะทางจุลกายวิภาคของอวัยวะสืบพันธุ์และคุณภาพอสุจิ อย่างไรก็ตามเมื่อหนูได้รับเคอร์ซีตินเป็นเวลานานขึ้น และขนาดสูงขึ้น พบว่าในกลุ่ม Q270 ที่ 7 และ 14 วันและในกลุ่ม Q90 ที่ 14 และ 28 วัน น้ำหนักอัณฑะและท่อพักเชื้ออสุจิ-ท่อนำเชื้ออสุจิและคุณภาพอสุจิเพิ่มขึ้น ในขณะที่น้ำหนักของต่อมลูกหมากและถุงน้ำอสุจิลดลง การเปลี่ยนแปลงของน้ำหนักสอดคล้องกับการเปลี่ยนแปลงลักษณะทางจุลกายวิภาคของอวัยวะสืบพันธุ์ และพบว่าการเพิ่มขึ้นของคุณภาพอสุจิสัมพันธ์กับการเพิ่มขึ้นของน้ำหนักอัณฑะและท่อพักเชื้ออสุจิ-ท่อนำเชื้ออสุจิ เกิดจากการกระจายของอสุจิและของเหลวภายในท่อ ระดับ LH ในเลือดลดลงในกลุ่ม Q90 ที่ 7, 14 และ 28 วันและ Q270 ที่ 3, 7 และ 14 วัน ระดับ FSH ในเลือดลดลงในกลุ่ม Q90 ที่ 7 และ 28 วันและ Q270 ที่ 7 และ 14 วัน และระดับเทสทอสเตอโรน (T) ในเลือดลดลงในกลุ่ม Q270 ที่ 3 และ 14 วัน เคอร์ซีตินทุกขนาดไม่มีผลต่อการแสดงออกของ mRNA ของ StAR โปรตีนและ P450scc ยกเว้นในกลุ่ม Q90 ที่ 28 วัน ที่พบว่าการแสดงออกของ mRNA P450scc ลดลง การลดลงของระดับเทสทอสเตอโรน มีความสัมพันธ์กับการลดลงของระดับ LH และ FSH แต่ไม่มีผลที่การสร้างสเตอรอยด์ฮอร์โมนที่เซลล์เลย์ดิก แสดงว่าเคอร์ซีตินอาจจะมีฤทธิ์เป็นแอนโดรเจนที่ไปยับยั้งการสร้างและหลั่งฮอร์โมนที่ไฮโปทาลามัส และต่อมใต้สมอง

จากการศึกษาผลของเคอร์ซีตินต่อการเจริญพันธุ์ หนูเพศผู้ที่ได้รับการฉีดเคอร์ซีตินเข้าทางใต้ผิวหนังขนาด Q0, Q90, Q270 เป็นเวลา 14 วัน และ 28 วันในกลุ่ม Q0 และ Q90 ระหว่าง 5 วันสุดท้ายของการทดลอง (ช่วงวันที่ 10-14 และ 24-28) ให้หนูทดลอง 1 ตัวอยู่กับหนูเพศเมียเต็มวัย 2 ตัว เพื่อตรวจความสามารถในการผสมพันธุ์กับตัวเมีย ซึ่งเป็นดัชนีบ่งชี้การเจริญพันธุ์ เมื่อหนูตั้งท้องครบ 14 วัน ทำการตรวจลอบการฝังตัวของตัวอ่อนในมดลูก พบว่าในกลุ่ม Q90 ที่ 28 วัน ไม่มีการเปลี่ยนแปลงของ ดัชนีบ่งชี้การเจริญพันธุ์ แต่ร้อยละของตัวผู้ที่พบอสุจิในหนูตัวเมียเพิ่มขึ้น และพบว่าในกลุ่ม Q270 ที่ 14 วัน มีการลดลงของดัชนีบ่งชี้การเจริญพันธุ์ ร้อยละของตัวผู้ที่พบอสุจิในตัวเมีย จำนวนตัวเมียที่ท้องและจำนวนตัวอ่อนในมดลูก การลดลงของภาวะเจริญพันธุ์อาจจะเกี่ยวข้องกับการเปลี่ยนแปลงของน้ำหนักและลักษณะทางจุลกายวิภาคของต่อมลูกหมากและถุงน้ำอสุจิและการลดลงของระดับเทสทอสเตอโรน

จากการศึกษาฤทธิ์ของเคอร์ซีตินต่ออวัยวะสืบพันธุ์และฮอร์โมนเพศในหนูแรทที่ถูกตัดอัณฑะออกเป็นเวลา 14 วัน หนูได้รับการฉีดเคอร์ซีตินเข้าทางใต้ผิวหนังในขนาด Q0, Q30, Q90 หรือ Q270 หรือสารละลายเทสโทสเตอโรนโปรปิโอเนต (Tp) ขนาด 1 มก/กก/วัน และกลุ่ม sham ได้รับ 20% glycerol เป็นเวลา 14 วัน พบว่าหนูที่ถูกตัดอัณฑะออกมีระดับ LH, FSH เพิ่มขึ้น ระดับเทสทอสเตอโรนลดลง และน้ำหนักของท่อพักเชื้ออสุจิ-ท่อนำเชื้ออสุจิ ต่อมลูกหมากและถุงน้ำอสุจิลดลง เคอร์ซีตินไม่สามารถทำให้ผลที่เกิดจากการตัดอัณฑะออกกลับคืนมาได้ เมื่อเทียบกับกลุ่ม Tp จากผลดังกล่าวแสดงว่าเคอร์ซีตินอาจจะไม่มีฤทธิ์เป็นแอนโดรเจนหรือถ้ามีก็มีเพียงเล็กน้อยในหนูที่ถูกตัดอัณฑะออก

จากผลการศึกษาที่ได้ในครั้งนี้ แสดงว่าเคอร์ซีตินอาจจะไม่มีฤทธิ์ในการเป็นแอนโดรเจนโดยตัวมันเอง แต่อาจจะมีฤทธิ์ในส่งเสริมการทำงานของแอนโดรเจนที่มีอยู่ในร่างกาย หรือผ่านทางกลไกอื่นเช่นการเป็นแอนติออกซิแดนท์ จากผลดังกล่าวน่าจะพิจารณาใช้เคอร์ซีติน (Q90) เป็นยาเพื่อรักษาภาวะความไม่สมบูรณ์ของการเจริญพันธุ์

สาขาวิชา สรีรวิทยา (สหสาขาวิชา) ลายมือชื่อนิสิต.....

ปีการศึกษา 2550 ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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LADACHART TAEPONGSORAT: EFFECTS OF QUERCETIN ON SPERMATOGENESIS AND STERIODOGENESIS IN INTACT AND ORCHIDECTOMIZED RATS. THESIS ADVISOR: ASSOC. PROF. PRAKONG TANGPRAPRUTGUL, Ph.D., THESIS CO-ADVISORS: ASSOC. PROF. SUCHINDA MALAIVIJITNOND, Ph.D., NOPPADON KITANA, Ph.D., 136 pp.

This thesis reports the effects of quercetin, a flavonoid found in many plant species including Thai herb used for sexual dysfunction treatment, on spermatogenesis and steroidogenesis of male rats.

Effects of quercetin on spermatogenesis and steroidogenesis were first studied in the intact male Sprague-Dawley rats. Eight weeks old rats were subcutaneously injected with quercetin at dose of 0 (Q0), 30 (Q30), 90 (Q90) or 270 (Q270) mg/kgBW/day for 3, 7 or 14 days, and 28-day treatments were performed additionally with Q0 and Q90 rats. The results showed that effects of quercetin on reproductive organs (testes, epididymis-vas deferens, prostate gland and seminal vesicle), sex hormones (testosterone, LH and FSH) and sperm quality (sperm concentration, viability and mobility) depend on dose and duration of treatment. The shortest time of quercetin treatment (3 days) at any dosage had no effects on reproductive organ weights and histological alterations and sperm quality. However, the prolonged (7, 14 and 28 days) and higher doses (Q90 and Q270) treatments affected the reproductive parameters. The weights of the testes and epididymis-vas deferens and sperm quality of Q270 (day 7 and 14) and Q90 (day 14 and 28) were significantly increased, while weights of prostate gland and seminal vesicle were significantly decreased. The weight changes were associated with the histological alterations of sex organs, while the increased sperm quality was related to the increased weights of testis and epididymis via the distribution of sperm and fluid. Serum LH levels (Q90 at day 7, 14 and 28; Q270 at day 3, 7 and 14) FSH levels (Q90 at day 7 and 28; Q270 at day 7 and 14) and testosterone levels (Q270 at day 3 and 14) were significantly decreased. The expression of mRNA of StAR protein and P450scc were not significantly changed after quercetin treatment, except that of the Q90 rats at day 28 which showed a reduced expression of P450scc. The decreased testosterone levels which associated with the reduced levels of serum LH and FSH, but not the Leydig cell steroidogenesis, indicated that quercetin may have an androgenic activity to suppress HPG axis.

Effects of quercetin on fertility were further studied in male rats subcutaneously injected with Q0, Q90 or Q270 for 14 days, and Q0 or Q90 for 28 days. During the last five day of treatment (day 10-14 or day 24-28), the treated male rat was housed with two virgin untreated female rats. Although there were no changes of fertility indexes (or the male ability to mate with virgin female) in Q90 rats even that the treatment was prolonged to 28 days, the percentage of male with sperm positive female was significantly increased. Fertility indexes, percentage of male with sperm positive female, number of pregnant females, and number of fetus per litter were significantly decreased in Q270 rats of 14-day treatment. The reduced fertility in the treated rats may be associated with changes in weight and histology of prostate gland and seminal vesicle and the reduced testosterone levels.

Actions of quercetin on reproductive organs and sex hormone levels were studied in orchidectomized (ODX) rats. The rats were subcutaneously injected with Q0, Q30, Q90 or Q270 or 1 mg/kgBW/day of testosterone proprionate (Tp) for 14 days. Sham group was subcutaneously injected with vehicle (20% glycerol). After ODX for 14 days, the rats showed increased FSH and LH levels, decreased testosterone levels and decreased weights of epididymis-vas deferens, prostate gland and seminal vesicle compared to the sham rats. Although Tp treatment could recover these effects, these changes were not recovered after quercetin treatment. The result indicates that quercetin may have very low or no androgenic activity in ODX rats.

Overall results suggest that quercetin may exhibit its androgenic activity by potentiating endogenous androgens or through other mechanisms such as the antioxidant effects. Based on these results, the use of quercetin (at Q90) as a drug for treatment of male infertility should be considered.

Field of study Physiology

Academic Year 2007

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LIST OF ABBREVIATIONS

ºC	= Degree Celsius
3β-HSD	= 3β-Hydroxysteroid dehydrogenase
17β-HSD	= 17β-Hydroxysteroid dehydrogenase
cAMP	= Cyclic adenosine monophosphate
cGMP	= Cyclic guanosine monophosphate
DHT	= Dihydrotestosterone
μl	= Microliter
FSH	= Follicle stimulating hormone
H&E	= Hematoxylin and eosin
HPG	= Hypothalamus-pituitary-gonadal axis
kgBW	= Kilogram body weight
LH	= Luteinizing hormone
MDA	= Malondyaldehyde
ml	= Milliliter
mRNA	= Messenger ribonucleic acid
ng	= Nanogram
nm	= Nanometer
OD	= Optical density
P450scc	= Cytochrome P450 side chain cleavage
RIA	= Radioimmunoassay
ROS	= Reactive oxygen species
RT PCR	= Reverse transcriptase polymerase chain reaction
s.c.	= Subcutaneous injection
StAR	= Steroid acute regulatory protein
T	= Testosterone
Tp	= Testosterone proprionate

CHAPTER I

INTRODUCTION

1.1 Rationale

Historically, medicinal plants or herbs are widely used to conquer diseases. Trend of using the herbal medicine instead of modern drugs has increased because people believe that drugs from natural resources have less side effect than synthetic ones. Moreover, systematic natural product researches have been developed in order to get phytochemicals which can be safely and effectively used for physical suffering treatment (Subcharoen, 1999).

The *Mucuna macrocarpa* Wallich (synonym; *Mucuna collettii* Lace), locally called the Black Kwao Krua, is one of the leguminous herbs that has been traditionally used by Thai males for the purposes of rejuvenation as well as maintenance of sexual performance or prevention of erectile dysfunction (Suntara, 1931). Several studies of the Black Kwao Krua on reproductive organs and its anti-estrogenic (or androgenic) effects had been carried out recently (Thansa, 2003; Cherdshewasart *et al*., 2004; Srijunngam *et al*., 2006). Feeding of 400 mg/kgBW/day of the Black Kwao Krua to rats for 30 days significantly increased testicular weight and tended to increase sperm numbers in caudal epididymis and testes (Wuteeraphon *et al*., 2001). However, feeding the suspension of the Black Kwao Krua (10-100 mg/kgBW/day) for 30 days had no effects on the weight and histopathology of testis, seminal vesicle and prostate gland in male rats (Thansa, 2003). Administration of 14 ppm of the crude extract of the Black Kwao Krua to male tilapia (*Oreochromis niloticus*) increased gonadosomatic index of fish after 4-5 month treatment but showed no indication of enhanced reproductive function after 6-7 month treatment

(Srijunngam *et al*., 2006). The Black Kwao Krua exhibited an anti-proliferative effect on the growth of MCF-7 cells, an estrogen receptor positive (ER^+) human mammary adenocarcinoma, which may suggest its anti-estrogenic function (Cherdshewasart *et al*., 2004). So far, effects of the Black Kwao Krua on male reproductive organs are still inconclusive. Moreover, evaluating the antiestrogenic or androgenic effects of the crude extract or the powder of the Black Kwao Krua directly may be not proper, as the Black Kwao Krua contains many bioactive substances. Thus, it is necessary to evaluate the effects of each substance of this plant.

By the analyses of extracts from the stem of the Black Kwao Krua, three bioactive substances were found: quercetin, kaempferol and hopeaphenol. These three substances function as a cyclic AMP phosphodiesterase inhibitor (Sookkongwaree, 1999). Of the three substances isolated, quercetin appeared to be a major one for research on male fertility and reproductive functions. In the *in vitro* study, quercetin inhibited the collective motility of ejaculated ram spermatozoa in the first two hours of incubation and stimulated it for the next three to four hours of incubation (Nass-Ardenand Breitbart, 1990). The incubation of human semen with quercetin induced an irreversible and dose dependent fall in sperm motility and sperm viability (Khanduja *et al*., 2001). The ejaculated bovine sperm motility was enhanced after quercetin administration (Hammerstedt *et al*., 1988). Quercetin did not affect cortisol production in human adrenal H295R cells stimulated with di-buthylyl cAMP (Ohno *et al*., 2002). Addition of quercetin to MA-10 mouse Leydig tumor cells resulted in an increase in the cAMP-stimulating progesterone secretion and the expression of StAR protein, a protein functioning in transferring of cholesterol from outer to inner membrane of mitochondria (Chen *et al*., 2007). The *in vivo* study in male rats reveals that two- time intraperitoneal injection of both 200 and 300

mg/kgBW of quercetin had no effects on fertility and the number of total and live implantations (Aravindakshan *et al*., 1985), but a gavages of quercetin at dose of 50-150 mg/kg BW/day for 10 days slightly increased prostate gland weights and dilated prostate lumens, which were full of secretory materials (Ma *et al*., 2004). Quercetin could increase serum testosterone levels and decreased serum dihydrotestosterone levels in male rats (Ma *et al*., 2004).

Considering the reports cited above, quercetin had the androgenic, estrogenic as well as antiestrogenic effects on male reproductive organs and effects are still controversial, perhaps because most of the previous studies were carried out *in vitro* using cell lines. We therefore studied the *in vivo* effects and mechanisms of quercetin on reproductive organs and hormones, sperm quality and fertility in adult male rats. Male rats were chosen for this study because of their small size, case of maintenance and thorough basic knowledge on their reproductive organs and functions (Semler, 1992; Rattanakorn, 2001).

1.2 Objectives

The aims of this study are

1. To study effects of quercetin on spermatogenesis (reproductive organs and sperm quality) in adult intact male rats.
2. To study effects of quercetin on steroidogenesis (serum gonadotropin and testosterone levels and the expression of StAR protein and P450scc) in adult intact male rats.
3. To study effects of quercetin on male fertility.

4. To investigate actions of quercetin on reproductive system (weight and histology of accessory sex organs and serum gonadotropin and testosterone levels) in orchidectomized rats

CHAPTER II

LITERATURE REVIEWS

2.1 Quercetin

2.1.1 Structure and Source

Quercetin belongs to an extensive class of polyphenolic flavoniod compounds (Figure 2.1). It is almost ubiquitous in plants and plant food sources. Red onions are the richest source of quercetin, and it is also found in tea, red apples (skin), berries, cherries, red and purple grapes, plums, and red wine (Lamson and Brignall, 2000). Quercetin is termed the aglycone or sugarless form of rutin. Frequently, it occurs as β–glycosides (sugar derivatives) (Herrmann, 1976; Brown, 1980). Its reputation as an antioxidant stems from the reactivity of phenolic compounds with free radical species to form phenoxy radicals, which are considerably less reactive. Additionally, polyphenolic compound is easily oxidizable to a quinoid form and participating in the redox reaction (Lamson and Brignall, 2000).

Data on the quercetin content in foodstuffs are limited. The available data suggest a range of 2-250 mg quercetin/kg wet weight in fruits; 0-100 mg/kg in vegetables, 200-600 mg in onions; 4-16 mg/L in red wine; 10-25 mg/L in tea and 2-23 mg/L in fruit juices (Hertog *et al*., 1992, 1993). Quercetin is the major bioflavonoid in the human diet. The estimated average dietary intake of quercetin in the Netherlands was 16 mg/day (Hertog *et al*., 1992), and in the United States was 25 mg/day (Lamson and Brignall, 2000).

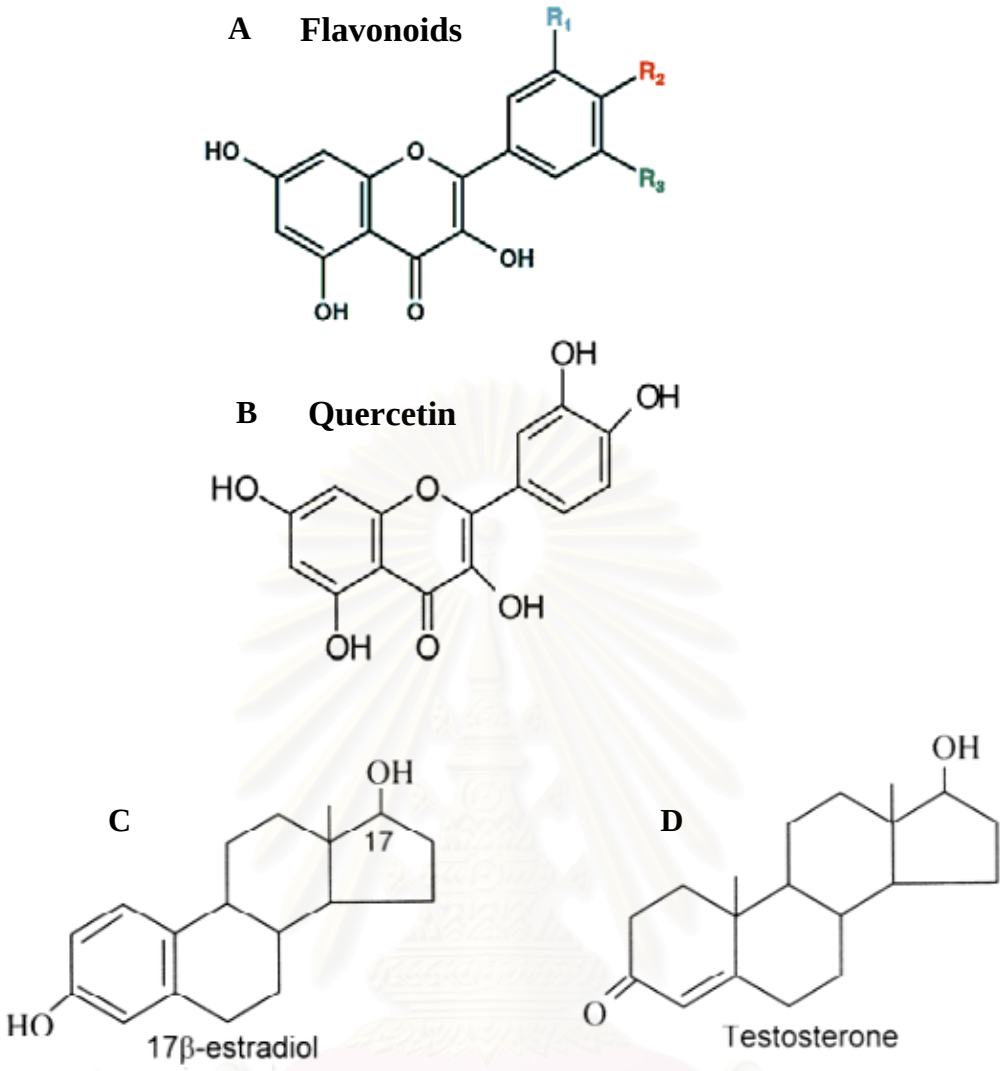

Figure 2.1 Structures of flavonoid (A), quercetin (B), estradiol (C) and testosterone (D).

Quercetin was also found in the stem of the Black Kwao Krua (Sookongwaree, 1999), one of leguminous herbs that Thai people used for improvement of male sexual functions (Roengsamran *et al*., 2001). In Thai traditional remedy, both women and men used this herb as the aphrodisiac (Suntara, 1931). Therefore, it became a subject for research about its effects on reproductive functions in these few years.

2.1.2 Pharmacokinetics of Quercetin

Quercetin, but not its glycosides, is absorbed from the rat stomach (Crespy *et al*., 2002), whereas quercetin glycosides are hydrolyzed in the small intestine by cleavage of the β–glycoside linkage by bacterial enzymes of colonic microflora (Griffiths and Smith, 1972; Walle *et al*., 2000). Microflora of the lower bowel hydrolyzes the flavonide-glycoside to quercetin and sugar, and quercetin is then absorbed into the enterohepatic system (Bokkenheuser *et al*., 1987). Quercetin is quickly metabolized in the liver (Gugler *et al*., 1975). In human, quercetin absorption is enhanced by conjugation with glucose (Hollman *et al*., 1995). The absorption of oral dosages of quercetin aglycone is approximately 20 percent in most animal and human trials (Murray *et al*., 1954).

Rats eating a diet supplemented with 0.2 % quercetin for three weeks attained a serum concentration of 133 μM of quercetin, mainly in sulfated and glucuronidated forms (Morand *et al*., 1998). Humans fed fried onions containing quercetin glycosides equivalent to 64 mg of the aglycone form reached a maximum serum concentration of 196 ng/ml (0.6 μM) of quercetin at 2.9 hr after ingestion. The half-life of this dose was 16.8 hr, and significant serum levels were noted up to 48 hr after ingestion (Hollman *et al*., 1996). In human, a single oral dose of 100 mg of quercetin led to a 0.8 μM quercetin in serum, however, a daily oral dose of 1500 mg led to a 10 μM quercetin in serum (Hollman *et al*., 1997).

After an oral administration, 20% of the dose was absorbed from the rat digestive tract and then excreted as glucuronide or sulfate conjugates into the bile and urine within 48 hr (Brown and Griffiths, 1983). After feeding rats with 5 mg of [^{14}C]quercetin, 80% of the total radioactivity was detected at the intestine content (Petrakis *et al*., 1959). The urinary excretion of quercetin or its conjugates accounted

for 4% of the dose (Petrakis *et al*., 1959), and ranged from 0.07 to 17.4% of intake (Morand *et al*., 1998). The fecal excretion of quercetin at up to 72 hr after dosing accounted for 53 ± 5 % of the dose and only traces of quercetin appeared in the feces after that (Gugler *et al*., 1975).

The major circulating metabolites of quercetin are glucurono-sulfo conjugates of isorhamnetin and quercetin (Morand *et al*., 1998). After oral administration of quercetin to rabbit (Booth *et al*., 1956) or rats (Petrakis *et al*., 1959), three metabolites of quercetin were identified in the urine: 3,4-dihydroxyphenylacetric acid, 3-methoxy-4-hydroxy-phenylacetic acid (homovanillicacid), and m-hydroxyphenyacetic acid. These metabolites were thought to be formed in the liver after fusion of the heterocylicpyrone ring. Administration of quercetin to rats by intraperitoneal injection, the 3'-o-methylether of quercetin (isorhamnetin) was identified as a metabolite in bile (Brown and Griffiths, 1983).

The plasma of quercetin in man following a single intravenous injection of 100 mg of quercetin led to a concentration of 12 μM as analyzed according to a two-compartment model with half-life of 8.8 ± 1.2 min (0-40 min) and 2.4 ± 0.2 hr (40-540 min) (Gugler *et al*., 1975). The apparent volume of distribution was 0.34 ± 0.03 liter/kg and protein binding in the plasma is estimated to be in excess of 98%. Only 0.65% of the intravenous dose was excreted unchanged and 7.4 ± 1.2 % was excreted in urine as conjugated metabolites. An oral dose of 4 g of quercetin in human volunteers (50-65 mg/kg) failed to detect the measurable plasma concentration and neither quercetin nor conjugated quercetin was detected in urine at any time. However, only quercetin glucuronides, not free quercetin, could be detected in plasma (Graefe *et al*., 2001).

2.1.3 Toxicology of Quercetin

A single oral dose of up to 4 g of quercetin was not associated with side-effects in human (Gugler *et al*., 1975) or twice daily feeding of 500 mg for 1 month to human did not show any adverse effects (Shoskes *et al*., 1999). Intravenous bolus of 2.5 g/ 70 kgBW once a week for 3 weeks was associated with a renal toxicity in two of ten patients. The two patients had a reduction in glomerular flow rate of nearly 20% in the first 24 hr. However, the reduction resolved within one week (Ferry *et al*., 1996). In an *in vitro* study, quercetin increased the cytotoxic effect of cyclophosphamide (Hofmann *et al*., 1988), and decreased resistance to gemcitabine and topotecan (Sliutz *et al*., 1996).

The oral LD_{50} of quercetin was reported as 160 mg/kg in mice and 161 mg/kg in rats. The LD_{50} of the subcutaneous injection of quercetin in the mouse was reported as 97 mg/kg (Sullivan *et al*., 1951). The reproductive toxicity of quercetin was studied in male and female F344 rats fed with diets containing 0.1% or 0.2% quercetin from birth to breeding during weeks 12 or 13 and fed with diets without quercetin during gestation and lactation. It was found that quercetin had no effect on mean viable litter size, live birth index, 3 day survival pups, lactation index, or weight of pups at birth or at 21 days (Stoewsand *et al*., 1984). Administration of 0, 20, 200, or 2000 mg/kg of quercetin to female Sprague-Dawley rats from days 6-15 of gestation showed no overt signs of toxicity in the dams even at the highest dose. However, the average fetal weight of the 2000 mg/kg group was reduced relative to the control fetal weight. Nevertheless, fetal abnormalities attributable to the chemical administration were observed (Willhite, 1982).

2.1.4 Benefits of Quercetin

Quercetin is one of the important antioxidant phytochemicals that are being investigated for its effects on a large number of health benefits. The antioxidant properties of quercetin can protect the integrity of human sperm DNA from reactive oxygen species (ROS) induced by estrogenic compounds (Cemeli *et al*., 2004). Other benefits specifically associated with quercetin include antihistamine, antiallergenic and anti-inflammatory action as well as reduce a risk of cancer, cardiovascular disease and cataracts (Lamson and Brignall, 2000). Quercetin is a highly effective antioxidant that has been shown to inhibit a free radical mediated damage of low-density lipoproteins *in vitro* (de Whalley *et al*., 1990).

Quercetin has a wide range of biological activities including inhibition of protein kinase C (Agullo *et al*., 1997), tyrosine kinase (Akiyama *et al*., 1987), phosphatidylinositide 3-kinase (PI-3 kinase) (Agullo *et al*., 1997) and DNA topoisomerase II (Constantinou and Huberman, 1995). Importantly, quercetin has antiproliferative activity *in vitro* against several cancer cells by inhibiting the expression of cyclin A (Yoshida *et al*., 1992), cyclin B1 (Choi *et al*., 2001), cyclin D1 (Kaneuchi *et al*., 2003) and Cdc2 (Yoshida *et al*., 1992; Choi *et al*., 2001). Quercetin inhibits the activities of several enzymes including cAMP-independent-protein kinase (Sharoni *et al*., 1984), calcium-phospholipid-dependent protein kinase (Gschwendt *et al*., 1984) and tyrosine protein kinase associated with mammary tumors (Levy *et al*., 1984), but has no effect on the activity of cAMP-dependent protein kinase (Graziani *et al*., 1983). Quercetin reduces the DNA damage produced in sperm and lymphocytes by 17β-estradiol, daizein, genistein and hydrogen peroxide (Cemeli *et al*., 2004). Quercetin also inhibits cell division and induces apoptosis through a pathway involving heat shock proteins (Wei *et al*., 1994). The action of quercetin is

probably mediated by interaction with type II estrogen binding sites (Ranelletti *et al*., 1992) or the aryl hydrocarbon receptor (Ashida *et al*., 2000).

2.2 Quercetin and Male Reproduction

The phenolic ring of flavonoids, *viz*. quercetin, was structurally similar to estrogens and testosterone (as shown in Figure 2.1), thus the flavonoid may bind to the estrogen and androgen receptors and possibly exerts estrogenic or anti-estrogenic activity (Le Bail *et al*., 1998) and androgenic or anti-androgenic activity. Quercetin may alter reproductive function via inhibition of cyclic adenosine monophosphate (cAMP) phosphodiesterase (Sookkongwaree, 1999) which may be related to a penile erection in human. However, cAMP pathway did not play a significant role in the penile erection in rats which mainly occur via cyclic guanosine monophosphate (cGMP) (Martinez-Pineiro *et al*., 1993).

2.2.1 Spermatogenesis and Quercetin

Spermatogenesis represents the process by which precursor cells termed spermatogonia undergo a complex series of divisions to give rise to spermatozoa (de Kretser and Kerr, 1994). This process takes place within the seminiferous epithelium which is a complex structure composed of germ cells and radially oriented supporting cells called Sertoli cells. The latter cells extend from the basement membrane of the seminiferous tubules to the lumen. The cytoplasmic profiles of the Sertoli cells are extremely complex as this cell extends a series of processes that surround the adjacent germ cells in an arboreal pattern (de Kretser and Kerr, 1994).

Due to the cyclic course of spermatogenesis, any given generation of the spermatogenic cells overlaps with an earlier or later generation to create a constant combination of cells known as cell associations or stages (Leblond and Clermont, 1952). The complete sequence of stage (14 stages in rats) constitutes one cycle of the seminiferous epithelium. The spermatogenic cycle is the process of spermatogenesis from a single spermatogonium type A generating a clone of spermatozoa at a constant and characteristic rate. In rat, the cyclic initiation of spermatogenesis occurs about 12 days. Spermatogenic cycle is one quarter of the 48-52 days required for completion of mature spermatozoa production. The duration of the cycle of the seminiferous epithelium in the normal Sprague-Dawley rat has been calculated as 12.9 days (Clermont and Harvey, 1965). The maturation of spermatogonia is regulated by LH and FSH in a stage-dependent manner (Parvinen, 1982).

Quercetin inhibited a collective motility of ejeculated ram spermatozoa in the first 2 hrs and then stimulated the motility for the next 3 to 4 hrs of incubation (Nass-Arden and Breitbart, 1990). In human semen samples, incubating with 5-200 μm quercetin resulted in an inhibition of sperm motility and sperm viability (Khanduja *et al*., 2001). This inhibition had been linked with decreased calcium ATPase activity (Breitbart *et al*., 1985; Khanduja *et al*., 2001). Quercetin also caused a rapid decrease in human sperm motility via the high concentration of intracellular calcium in caput epididymis (William and Ford, 2003). During the first mitotic prophase of early primary spermatocyte, quercetin inhibited the activity of a cytosolic calcium-magnesium ATPase and caused an increase in the intracellular calcium activity in human spermatozoa (Trejo *et al*., 1995). The *in vivo* study in male rats reveals that two-time intraperitoneal injection of 200 and 300 mg/kgBW of quercetin

had no effects on fertility and the number of total and live implantations (Aravindakshan *et al*., 1985).

2.2.2 Steroidogenesis and Quercetin

A common feature of steroid hormones is that their synthesis rely on cholesterol as a common precursor. The delivery of cholesterol to the site of its first enzymatic conversion constitutes the rate-limiting and hormonally regulated step in steroidogenesis (Karaboyas and Koritz, 1965). Cellular cholesterol residing in the outer mitochondrial membrane, lipid droplets or plasma membranes of steroidogenic cells, must be delivered to the inner mitochondrial membrane which is the site of the cytochrome P450 side chain cleavage enzyme (P450scc). The enzyme help convert cholesterol to pregnenolone, the first steroid formed in all steroidogenic tissues (Figure 2.2) (Garren *et al*., 1966).

It is well established that Leydig cell steroidogenesis is regulated by LH/chorionic gonadotropin and involves the increase in the formation of the second messenger cAMP signal transduction pathway via the LH receptor and guanosine-stimulated protein (Gs protein). Cyclic AMP then increases the formation of cholesterol from cholesterol esters and the conversion of cholesterol to pregnenolone via the activation of protein kinase A (PKA) to activate the protein synthesis. LH increases the gene transcription of steroidogenic acute regulatory (StAR) protein and several steroidogenic enzymes (de Kretser and Kerr, 1994; Payne *et al*., 1996).

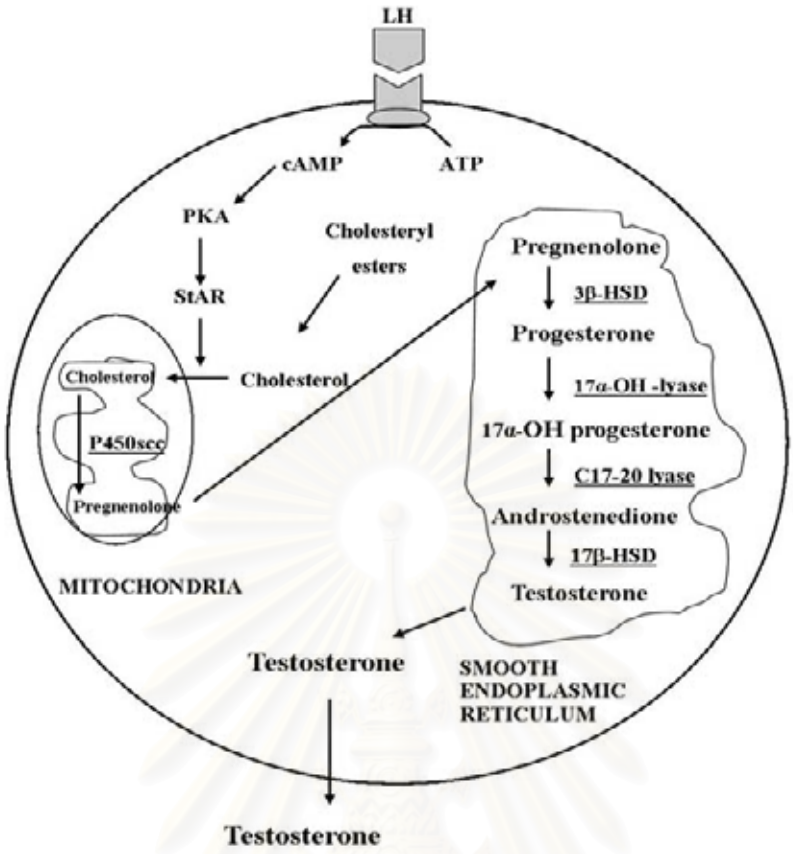

Figure 2.2 Illustration of the molecular events involved in testosterone production by Leydig cells. (Modified from Zirkin and Chen, 2000).

StAR protein (30-kDa; Clark *et al*, 1994) is a family of mitochondrial phosphoprotein found in adrenal and gonadal steroidogenic cells. It is rapidly synthesized when stimulated by tropic hormone such as cAMP, LH and FSH (Stocco and Clark, 1996a) and then imported into the mitochondria of steroidogenic cells. StAR import requires the presence of an electrochemical gradient across the inner mitochondrial membrane (Stocco and Sodeman, 1991). The StAR protein functions in transfer of cholesterol from outer to the inner mitochondrial membrane which is the rate-limiting and acutely regulated step in steroidogenesis (Clark *et al*., 1994; Stocco and Clark, 1996b; Pollack *et al*., 1997).

The conversion of cholesterol to testosterone in mitochondria involved a number of steps that are catalyzed by enzymes, predominantly belonging to

cytochrome P450 family. The initial step is the conversion of cholesterol to pregnenolone by the P450scc located at the inner membrane of mitochondria. Pregnenolone diffuses across the mitochrondrial membranes and is further metabolized by enzymes associated with the smooth endoplasmic reticulum. In the mouse Leydig cell, pregnenolone is converted to progesterone by the action of 3β-HSD. The next reaction is catalyzed by the cytochrome P450 enzyme, 17α hydroxylase, a single protein that catalyzed the hydroxylation of progesterone to yield the androstenedione, the immediate precursor of testosterone. The final reaction is the reduction of the 17 ketone of androstenedione by a microsomal 17β hydroxysteroid dehydrogenase (17β HSD) enzyme (Payne and Youngblood, 1995). Testosterone can be converted to a dihydrotestosterone (DHT) by the 5α reductase enzyme (Andersson and Russel, 1990) or can be metabolized to 17β estradiol by the aromatase enzyme (Chen *et al*., 1988).

A daily gavage dose of 50, 100 or 150 mg/kgBW of quercetin for 10 days in rats showed significantly increased serum testosterone levels (Ma *et al*., 2004). Ma and co-worker (2004) found that quercetin resulted in a biphasic change in serum DHT concentration, which was slightly increased at a low dose (50 mg/kgBW) and decreased at a high dose (150 mg/kgBW). However, the incubation of quercetin (12.5 μM) in adrenocortical H295R cells did not change the cortisol production (Ohno *et al*., 2002). Addition of quercetin to MA-10 mouse Leydig tumor cells increased the cAMP-stimulating progesterone secretion and the expression of StAR protein (Chen *et al*., 2007).

Quercetin is a weaker inhibitor, inhibiting the aromatization of androstenedione and testosterone to estradiol in primary cultures of human granulosa-luteal cells (Lacey *et al*., 2005) and human placental and ovarian microsomes (Kellis

and Vickery, 1984). The incubation of high dose of quercetin in primary cultures of human granulosa-luteal cells inhibits aromatase/17β hydroxysteroid dehydrogenase (17β-HSD) activities (Lacey *et al*., 2005). However, quercetin did not inhibit activity of 17β-HSD type 1 in breast cancer cells (Makela *et al*., 1995).

The conversion of androstenedione to estradiol may either go via estrone involving both aromatase and 17β-HSD type 1 or via testosterone involving 17β-HSD type 5 and aromatase, respectively. Quercetin was the most potent flavonol inhibiting the reductive activity of recombinant 17β-HSD type 5 and relatively potent inhibitors of 17β-HSD type 1 in placental microsomes (Krazeisin *et al*., 2001). Quercetin was found to be potent inhibitors of the aromatase enzyme complex from rainbow trout (Pelissero *et al*., 1996), and aromatase activity in colorectal cancer cell lines (Fiorelli *et al*., 1999).

2.3 Actions of Gonadotropins and Androgens on Male Reproduction

Peptide and steroid hormones are critical not only for regulation of male germ cell development, but also for the proliferation and function of the somatic cell types which are required for a proper development of the testis (Sharpe, 1994; McLachlan *et al.*, 2002). The interstitial steroidogenic Leydig cells appear to have a primary function of testosterone production (Mendis-Handagama, 1997). The Sertoli cells are essential for providing both physical and nutritional support for spermatogenesis (Griswold, 1998). Each of these cell types is a direct target for one or more of the hormones whose actions are essential for unimpaired male fertility. Both FSH and testosterone are necessary for the initiation of spermatogenesis. However, once the initial stimulation of FSH has occurred, testosterone alone can maintain spermatogenesis for a long time thereafter (Ross *et al*., 1995).

FSH targets primarily the Sertoli cells of the seminiferous tubules. Under FSH stimulation, and in the presence of testosterone from the Leydig cells, Sertoli cells promote spermatogenesis and spermiogenesis. The rate of spermatogenesis is regulated by a negative feedback mechanism involving gonadotropin releasing hormone (GnRH), FSH, and inhibin. Under GnRH stimulation, FSH promotes spermatogenesis along the seminiferous tubules. Inhibin secretion by Sertoli cells may accelerate the spermatogenesis (Martini and Bartholomew, 1997). Inhibin inhibits FSH production in the anterior pituitary (Figure 2.3) (Griswold, 1998). FSH also increases the synthesis of structural proteins and an androgen-binding protein (ABP) by Sertoli cells which is secreted into the extracellular fluid surrounding the cells of the germinal epithelium (Davies, 1981).

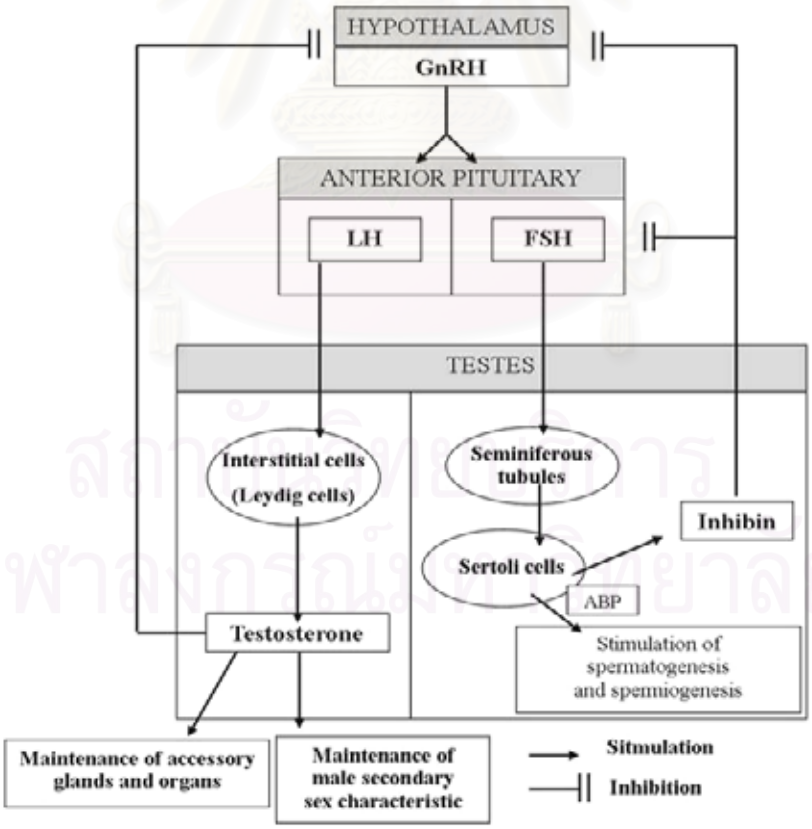

Figure 2.3 Hormonal feedback and the regulation of male reproductive function. (Modified from Martini and Bartholomew, 1997).

Testosterone, the most important androgen, has numerous functions. It promotes the functional maturation of sperm, maintains the accessory organs of the male reproductive tract, determines the secondary sexual characteristics such as the distribution of facial hair, increases muscle mass and body size, and the quantity and location of characteristic adipose tissue deposits, stimulates metabolic operations throughout the body, influenced brain development and stimulates sexual behaviors and sexual drive (Martini and Bartholomew, 1997). Testosterone can also regulate its synthesis via negative feedback mechanism at two levels of hypothalamus-pituitary-gonadal (HPG) axis (Figure 2.3) (Martini and Bartholomew, 1997).

Testosterone is secreted into the blood circulation by binding to carrier proteins (β-globulin and albumin), then transported into the blood or lymphatic capillaries or through the peritubular tissue and finally entered the seminiferous tubules. Like other steroid hormones, testosterone acts via the nuclear receptor in its target tissue. In many target cells such as prostate gland, seminal vesicle and male external accessory organs, testosterone is converted to another androgen called dihydrotestosterone (DHT) before exerts its action. During the transport of androgens, some molecules are changed by the liver into forms that can be excreted in bile or urine (Cunha *et al*., 1987; Hole, 1999).

Upon elimination of testicular testosterone production, the maintenance of testis weight required a circulating androgen at much higher level than the need to maintain accessory sex organ weights. Testes, prostate, and seminal vesicles are equally stimulated when the androgen receptor in these tissues is exposed to the same intracellular concentration of free androgen, and the low 5α reductase activity in the testis plays a critical role in the differential response of the testis and the accessory sex organs to testosterone (Turner *et al*., 1984).

Spermatogenesis requires a relatively higher level of testicular androgens compared to the circulating level of androgens that can evoke a normal response in other androgen dependent tissues (Sun *et al*., 1989; Santulli *et al*., 1990). DHT has a 2-fold greater affinity for the androgen receptor and a one-fifth dissociation rate compared with that of testosterone, resulting in formation of a more stable ligand receptor complex (Grino *et al*., 1990). DHT can maintain a testis weight at a lower dose than testosterone (Chen *et al*., 1994). The DHT level needed to maintain qualitatively normal spermatogenesis is about 50% of testosterone level. In human, DHT is crucial for the development of the male reproductive tract. However, in male mice, DHT plays little or no role in the development (Walsh *et al*., 1974; Mahendroo *et al*., 2001).

Testosterone is essential for growth and division of the spermatogonia in the formation of sperm. Estrogens, formed from testosterone by the Sertoli cells under the influence of FSH are probably essential for spermiogenesis. The Sertoli cells also secrete an androgen-binding protein that binds both testosterone and estrogens and carry these into the fluid in the seminiferous tubular lumen, making these hormones available to the maturing sperm.

Estrogens are also essential for proper sexual differentiation and the maintenance of normal spermatogenesis in male. Estrogens increase the level of sex hormone-binding globulin that bind testosterone, resulting in lower level of free testosterone thereby decreasing androgenic stimulation of the prostate (Vij and Kumar, 2004).

Androgen receptor activity is regulated by testosterone and DHT. Binding of testosterone or DHT to an androgen receptor initiates nuclear translocation and the transcriptional regulatory function of androgen receptor (Lindzey *et al*., 1994).

Androgen receptor itself plays an important role in the regulation of testosterone levels through an autocrine feedback on the Leydig cells, via endocrine effects on GnRH production, and through inhibition of LH synthesis and secretion by the pituitary (Amory and Bremner, 2001).

2.4 Quercetin as Antioxidant in Male Reproduction

Quercetin works as an antioxidant by scavenging free radicals. These particles occur naturally in the body but can damage cell membranes and interact with genetic material. Quercetin was a highly effective antioxidant that has been shown to inhibit free radical mediated damage of low-density lipoproteins *in vitro* (de Whalley *et al*., 1990).

Quercetin induced a dose-dependent decrease in concentration of malondialdehyde (MDA) which is the product of lipid peroxidation (LPO). Quercetin showed the most potent antioxidant activity *in vitro*, even when compared to that of E_2. The compared with effective concentration to decrease 50% of MDA levels of quercetin was 3.4 uM/l, 29 uM/l of E_2 and 280 uM/l of genistein (Arteara *et al*., 2004).

Chronic administration of quercetin (10 mg/kgBW for 5 wks) to spontaneously hypertensive rats increased glutathione peroxide activity, decreased hepatic and plasma MDA levels. Thus, in this genetic model of hypertension, quercetin showed both antihypertensive and antioxidant properties (Duarte *et al*., 2001).

Human sperm plasma membrane has a high content of polyunsaturated fatty acid which causes the membranes to be sensitive to a damage by free radicals (Alvarez *et al*., 1987). Peroxidative damage induced by reactive oxygen species (ROS) has been proposed as one of the major causes of defective sperm function. It

was shown that loss of motility in sperm samples related to the accumulation of ROS in the spermatozoa (Erasmus *et al*., 1992). Under the oxidative stress, spermatozoa may not be able to undergo an acrosomal reaction (Rao *et al*., 1989; Aitken and West, 1990). This results in a decreased capacity for sperm-oocyte fusion and concomitant loss of fertility (Plante *et al*., 1994).

Reactive oxygen species, such as the superoxide anion, hydrogen peroxide, hydroxyl radical and hypochlorite radical, are continuously produced in ejaculates by spermatozoa (Khanduja *et al*., 2001). Hydrogen peroxide is responsible for most of the damage to spermatozoa (Gagnon *et al*., 1991). Hydrogen peroxide does not affect sperm viability but caused sperm immobilization. It acts mostly via depletion of intracellular adenosine triphosphate and the subsequence decrease in the phosphorylation of axonemal proteins (Agarwal *et al*., 1994; 2005). ROS production in semen is inversely correlated with motility (de Lamirande and Gagon, 1992).

Hydrogen peroxide could diffuse across the membranes into the cells and inhibited the activities of some enzymes such as glucose 6 phosphate dehydrogenase. It leads to a decrease in the production of NADPH and a concomitant accumulation of GSSG (reduced form of glutathione), GSH (oxidized form of glutathione), and superoxide dismutase. This causes a decrease in the antioxidant defenses of the spermatozoa which ultimately leads to the peroxidation of the membrane phospholipids by ROS (Griveau *et al*., 1995). Hydrogen peroxide changes the membrane fluidity and integrity by accumulation of lipid peroxide and disrupts the ionophore-induced acrosome reaction and sperm motility. Further, the sperm antioxidants are located in the midpiece region, and hence are not in a position to protect the rest of the sperm plasma membrane (Aitken, 1994).

Normally, Leydig cells are particularly susceptible to extracellular sources of ROS because of their close proximity to testicular interstitial macrophages (Hales *et al*., 1999). ROS are produced continuously in most cells as a by product of mitochondria and microsomal electron transport and other metabolic process (Hales, 2002).

The cytochrome P450 enzymes of the steroidogenic pathway use molecular oxygen and electrons transferred from NADPH to hydroxylate the substrate (Hall, 1994). In this process, superoxide anion or other oxygen free radicals can be produced as a result of electron leakage in normal reaction or due to interaction of steroid products and other pseudosubstrates with the enzyme (Hornsby *et al*., 1983). Hydrogen peroxide, a potent ROS, inhibits steroidogenesis in Leydig cells (Diemer *et al*., 2003) and MA-10 tumor Leydig cells (Stocco *et al*., 1993).

Human spermatozoa are endowed with antioxidant enzymes, namely catalase (Jeulin *et al*., 1989), glutathione peroxidase (Alvarez and Storey, 1984) and superoxide dismutase (Menella and Jones, 1980). The activities of the peroxide-metabolizing enzymes, catalase and glutathione peroxidase are very low in germ cells (Yoganathan *et al*., 1989). Induction of superoxide dismutase has also been observed in mice Leydig cells (Kumar *et al*., 1990) and rat ovary (Laloraya *et al*., 1988) after LH injections.

CHARPTER III

MATERIALS AND METHODS

3.1 Animals

Male (220-250 g BW) and female (200-220 g BW) Sprague-Dawley rats at 7 weeks old were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. They were allowed to acclimatize to the laboratory environment for one week prior to the experiment and were used for this study at 8 weeks old. They were housed with 5 animals per stainless steel cage with sawdust bedding in a room under temperature (25 ± 1°C) and photoperiod (12:12 light/dark cycle) control at the Primate Research Unit, Chulalongkorn University, Bangkok, Thailand. They were given free access to water and rat chow (SWT Co, Ltd, Thailand). All experiments were performed between 08.00-11.00 a.m. The experimental protocol was approved by the Animal Ethical Committee in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University.

3.2 Quercetin Preparation

Quercetin powder (Sigma, St. Louis, MO, USA) was dissolved and diluted with 20% glycerol in 0.9% normal saline, mixed vigorously and stored in a dark bottle at 4°C. The quercetin solution was freshly prepared on a weekly basis.

3.3 Experimental Protocol

The LD_{50} of quercetin by the oral route was reported to be 160 mg/kg in mice and 161 mg/kg in rats (Sullivan, 1951). Thus, the initial dose of quercetin chosen for

this study is 30 mg/kgBW/day (approximately 1/5 of LD_{50}). Besides, it was reported that the intraperitoneal injections of 200 and 300 mg/kgBW of quercetin to male rats did not show any impairment on fertility (Aravindashan *et al*., 1985). Therefore, the other two doses of quercetin treatment in this study are raised to 90 and 270 mg/kgBW/day.

The experiment was separated into 3 parts as follows;

Experiment I: Effects of quercetin on spermatogenesis (reproductive organs and sperm quality), steroidogenesis (serum gonadotropin and testosterone levels) and its mechanisms (the expression of StAR protein and P450scc) in intact male rats.

One hundred twenty male rats were randomly divided into 4 groups (30 rats/group) and subcutaneously injected with quercetin at dose of 0, 30, 90 or 270 mg/kg BW/day at volume 0.4 ml (hereafter abbreviated Q0, Q30, Q90 and Q270, respectively). Each dose group was further divided into 3 subgroups (10 rats/group) and treated with quercetin for 3, 7 and 14 days.

Because the effects of quercetin on spermatogenesis and steroidogenesis were first observed in rats after Q270 treatment for 7 and 14 days and in rats after Q90 treatment for 14 days, therefore, it was questioned what could happen on spermatogenesis and steroidogenesis when the duration of Q90 treatment is extended. Thus, another 2 groups of rats subcutaneously injected with Q0 and Q90 (10 rats/group) for 28 days were set up.

Rats were weighed on the first and last day of study period. At the end of treatment period all groups of rats were anesthetized and blood samples were collected and the serum was separated and determined for LH, FSH and testosterone levels by radioimmunoassay (RIA) technique. Then the rats were euthanized by diethyl ether, and the testes, epididymis-vas deferens, prostate glands and seminal

vesicles were dissected and weighed. After weighing, left testis, epididymis-vas deferens, prostate glands and seminal vesicles were cut into pieces of 1 cm^3 and kept in Bouin's fixative for histopathological examination. Fresh epididymis and vas deferens were also kept for sperm quality analysis. Right testis was separated for steroidogenic regulatory protein (StAR) and cytochrome P450 side chain cleavage enzyme (P450scc) mRNA analysis by RT-PCR technique.

Experiment II: Effects of quercetin on fertility in male rats.

From the Experiment I, the effects of quercetin on spermatogenesis and steroidogenesis could be clearly observed only after Q90 treatment for 14 and 28 days and Q270 treatment for 14 days. In Experiment II, fifty male rats were divided into 5 groups (10 rats/group) and treated with quercetin at dose of 0, 90 or 270 mg/kgBW/day for 14 days, and at dose of 0 and 90 mg/kgBW/day for 28 days. During treatment (day 10-14 or day 24-28), the treated male rat was housed with two virgin untreated female rats for 5 days (a complete estrous cycle). To confirm the mating, sperm plug at vagina was checked every morning during those 5 days. If the sperm plug was not observed, the vaginal smear was performed to confirm the mating. Female rat that had sperm plug or sperm in vagina, an indicator of positive mating, was separated from the male and the date was defined as day 0 of gestation. All female rats were euthanized by diethyl ether on day 14 of gestation. The uteri were removed and examined for number of live and dead fetuses and number of resorptions.

The percentage of males with sperm-positive females was calculated as the number of male with either 1 or 2 sperm-positive females per the total number of male in that treatment group. The percentage of male fertility index was calculated as

the number of males with either 1 or 2 pregnant females per the total number of male in that group. This value also included pregnant females that had not been previously observed the sperm-positive smears. Number of male pregnating was the number of male that produced 0, 1 or 2 pregnant females. The percentage of resorptions was calculated as the number of resorptions per the total number of fetuses.

Experiment III: Actions of quercetin on reproductive organs and serum hormone levels in orchidectomized rats.

Sixty male rats were subjected to bilateral orchidectomy or sham operation. For orchidectomy, the rats were anesthetized by diethyl ether and the scortum were cut open. Testicle coverings were dissected to expose the testis and spermatic cord. The cord was tied and cut, the testes were removed and the skin was stitched. Sham operated rats were operated in the same way as for orchidectomized rats except that the testes were not removed.

After 14 days of operation, the orchidectomized rats were divided into 5 groups (10 rats/group) and subcutaneously injected with quercetin at dose of 0, 30, 90 or 270 mg/kg BW/day or 1 mg/kgBW/day of testosterone proprionate (Tp) for 14 days (Tobin *et al*., 1997). Sham operated group was subcutaneously injected with vehicle (20% glycerol). After 14 days of treatment, blood samples were collected and euthanized by diethyl ether. The epididymis-vas deferens, prostate gland and seminal vesicle were dissected and weighed. Epididymis-vas deferens, prostate gland and seminal vesicle were cut into pieces of 1 cm^3 and kept into Bouin's fixative for histopathological examination. Blood samples were determined for LH, FSH and testosterone levels by RIA techniques.

3.3.1 Blood Sample Collection

The rats were anesthetized by diethyl ether and collected approximately 5 ml of blood by closure cardiac puncture during 9.00-11.00 a.m. Blood samples were centrifuged at 2,500 rpm 4°C for 20 min. Serum was separated, aliquoted and stored at -20 °C until assayed for FSH, LH and testosterone levels.

3.3.2 Histological Examination

After the overnight fixation of testis and accessory sex organs in Bouin's fixative, tissues were dehydrated in a series of ethanol gradients and cleared in xylene. Tissues were then embedded in paraffin, microtomed into 6 μm sections and stained with hematoxylin and eosin (see Appendix II). Permanent preparations of all tissues were histologically examined and photographed using a digital camera (Canon, USA) mounted on a light microscope (Carl Zeiss, Germany).

Digital images of seminiferous tubules, epididymides and vas deferens were examined with a digital image analysis program (Image Pro Express, Media Cybernetics, Silver Spring, MD). Seminiferous tubular area was measured and averaged from 200 tubules per rat (10 rats/group). The tubular area, luminal area and tubular thickness of the epididymis were measured and averaged from 100 tubes per rat (10 rats/group). The diameter of vas deferens were measured and averaged from 5 sections per rat (10 rats/group).

3.3.3 Sperm Quality Analysis

After sacrifice, rat epididymis and vas deferens were removed. The caudal epididymis and vas deferens were squeezed with pairs of fine forceps and the contents of these structures were subjected to sperm quality analysis.

Sperm quality was determined by three parameters: sperm concentration, motility and viability. **Sperm concentration** was analyzed by the haemocytometer method (WHO, 1999). Sperm suspensions from the caudal epididymis and vas deferens were diluted 1:20 with Baker's solution and transferred into microcentrifuge tubes. The diluted samples were put into the counting chamber and the number of sperm was counted using a haemocytometer with improved doubles Neubauer ruling under a light microscope. The sperm concentration was expressed as million/ml. **Sperm motility** was analyzed and averaged by counting the motile and non-motile spermatozoa and expressed as the percent motility. **Sperm viability** was analyzed by the Trypan blue staining method (WHO, 1999). The nonviable spermatozoa which were stained blue and the viable ones which were unstained were counted under the light microscope. The viability of sperm was expressed as the percentage of viable sperm.

3.3.4 Hormone Analysis

Serum FSH, LH and testosterone levels were determined by RIA methods. Serum concentrations of LH and FSH were measured using National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) RIA kits for rat LH and FSH, as described previously (Bast and Greenwald, 1974; see Appendix III). The antiserum used were anti-rat LH (S-10) and FSH (S-11). Results were expressed in terms of NIDDK rat FSH (RP-2) and LH (RP-2). The percentage of inter-assay coefficient of variation of LH and FSH were 6.3 and 9.7, respectively. The percentage of intra-assay coefficient of variation of LH and FSH were 4.9 and 4.4, respectively.

Serum testosterone concentration was determined by a double-antibody RIA system using ^{125}I-labeled radioligand, as described previously (Taya *et al*., 1985; see Appendix III). The results obtained were expressed in terms of testosterone reference standards. The percentage of inter-assay and intra-assay coefficient of variation were 7.2 and 6.3, respectively.

3.3.5 Isolation of Total Ribonucleic Acid (RNA) from Leydig Cells

Right testis was removed and decapsulated. The decapsulated testis was gently dispersed in ice-cold 0.9 % sodium chloride (NaCl) with magnetic stirrer for 20 minute. Then the solution was filtered by Nylon membrane and centrifuged at 2,500 rpm 4°C for 10 minutes. The pellet of Leydig cells were pipetted and preserved in RNA Later Reagent (Reagent for RNA preservation) (Ambion, catalog#7024, The RNA Company, Japan) in microcentrifuge tube and kept in refrigerator at 4°C for overnight and then kept at -20°C before RNA extraction.

Total RNA was isolated by RNA Extraction kit (e-Zi RNA extraction kit, SunolinCorporation, Thailand). Leydig cells suspension of approximately 200 µl was homogenized in liquid N_2 and mixed with 500 µl of RA buffer and 500 µl of RB buffer, stood at room temperature for 5 min, then added 250 µl of RA buffer. The lysate was mixed for 2 min and then centrifuged at 13,000 rpm for 5 min. The cleared lysate was added with 500 µl of RC buffer, mixed for 20 sec and centrifuged at 13,000 rpm for 5 min. The supernatant was added with 500 µl of RD buffer and mixed by inverting for 5-6 times and incubated at 4°C for 10 min, then centrifuged at 13,000 rpm for 10 min. The pellet was dissolved by 500 µl of RE buffer and centrifuged at 13,000 rpm for 10 min. The pellet was washed by 500 µl of 70% ethanol and centrifuged at 13,000 rpm for 5 min. The pellet was air-dried and

dissolved again by 20 µl of nuclease-free water. The yields of obtained total RNA were determined by spectrophotometry (Spectrophotometer DU600; Beckman Coulter™, USA) at 260 nm, where one absorbance unit (OD260) equals 40 µg of single-stranded RNA/ml. The purity was estimated by spectrophotometry from the relative absorbance at 260 and 280 nm. The expected range of OD260/OD280 ratio for RNA should be 1.8-2.0. The purified total RNA was stored at -20°C.

3.3.6 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using Maxime RT-PCR PreMix kit (catalog# 25131, iNtRON Biotechnology, Korea). The reaction mixture of 25 µl comprised of Maxime RT-PCR PreMix (OptiScript™ RT System, RT-PCR buffer, dNTPs and i-StarTaq™ DNA Polymerase), 10 µM of Forward primer, 10 µM of Reverse primer, 200 ng RNA template and nuclease-free distilled water. Target cDNA was amplified with ribosomal protein S16 (RPS16) as the internal control in an aliquot of the synthesized product. Primers for the target cDNAs based on rat sequences were synthesized on an oligonucleotide synthesizer and shown in Table 3.1 (BioService Unit, Thailand). The expected product size was 399, 389 and 148 base pairs (bp) for P450scc, StAR and RPS16, respectively. RT-PCR was performed under the optimum condition as follows: 1 cycle of 37°C for 75 min and 94°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min; and 1 cycle of final extension at 72°C for 7 min (Akingbemi *et al*., 2000). After PCR amplification, PCR product was examined on 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining, and quantified on a densitometer (QuantityOne software, California, USA), using 100 bp DNA ladder as a

marker. The quantification of PCR products for StAR and P450scc was normalized to RPS16.

Table 3.1 Primers for StAR, P450scc and RPS16 genes.

Gene	Forward/Reverse Primer	Expected Product (bp)	Reference
StAR	5'-TTGGGCATACTCAACAACCA-3' 3'-GACTCGTTTCGCCACAGTA-5'	389	Furukawa *et al*., 1998
P450scc	5'-AGGTGTAGCTCAGGACTTCA-3' 3'-CCACAGGAAATATCGGAGGA-5'	399	Oonk *et al*., 1989
RPS16	5'-AAGTCTTCGGACGCAAGAAA-3' 3'-GACAAGACGAAGACCCGT T-5'	148	Chan *et al*., 1990

3.4 Statistical Analyses

The results were expressed as mean ± S.E.M. The relative organ weight (%) were obtained by the division of the organ weight by the body weight x 100. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) program version 11 (SPSS Inc., USA). Dose responses and time responses were analyzed by one-way analyses of variance (ANOVA) for factorial and repeated measure design with post-hoc testing by the least significant difference (LSD) test. The correlation between times and treatment groups was analyzed by two-way ANOVA. The P values of less than 0.05 were considered as significant difference.

CHARPTER IV
RESULTS

4.1 Effects of age on reproductive organs, sperm quality and serum reproductive hormone levels in intact male rats

4.1.1 Effects of age on body weight

With an advancing age from 8 to 12 weeks old, the body weights of Q0 rats were significantly increased (R^2 = 0.941) (Figure 4.1). The body weight gain started at 9 weeks old (or 7 days of study period). The gain of body weight was by approximately 1.2 times from 8 to 12 weeks of age.

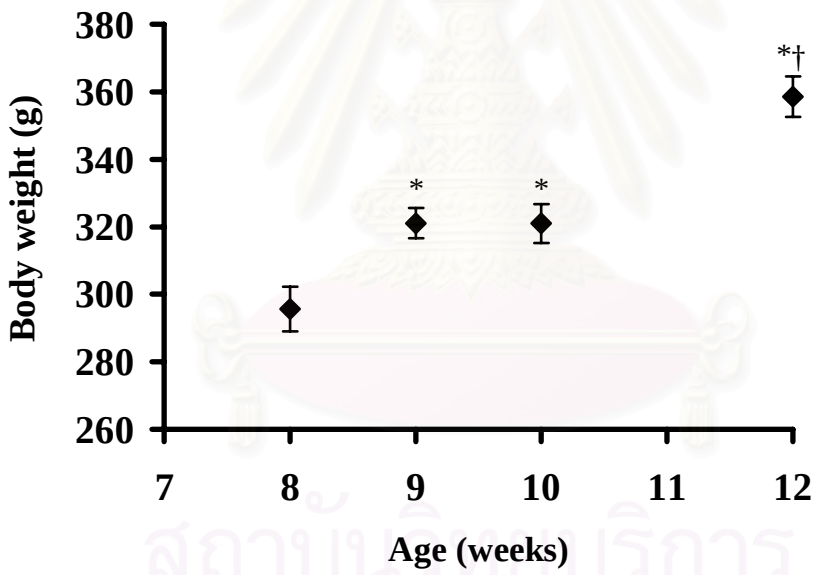

Figure 4.1 Body weight gain of male rats during 8-12 weeks old. * = significant difference ($p<0.01$) compared with 8 weeks. † = significant difference ($p< 0.01$) compared with 10 weeks and 9 weeks.

4.1.2 Effects of age on testis weight

The testis weight of control rats did not increase during the first 2 weeks of the study period (8-10 weeks) and significantly increased during the next 2 weeks (or 12 weeks of age) (Figure 4.2 A). The gain of testis weight was by approximately 1.2 times from 8 to 12 weeks of age.

4.1.3 Effects of age on weights of accessory sex organs

There was a positive, linear relationship between age and weights of accessory sex organs (epididymis-vas deferens, prostate glands and seminal vesicles) in the control rats (Q0) (Figure 4.2 B). The weight gain of accessory sex organs started at 7 days of study period (or 9 weeks of age). Epididymis-vas deferens weights were found to significantly increase up to 12 weeks of age of rats (or 28 days of experiment), whereas the prostate gland and seminal vesicle were found to be mature at 10 weeks of age (or 14 days of experiment). The latter 2 organs seemed to reach a plateau at 10 weeks of age. The gain of epididymis, prostate and seminal vesicle weight were by approximately 1.7, 1.7 and 1.8 times, respectively from 8 to 12 weeks of age.

Because the patterns of the body weight and organ weight gains were different, the relative organ weights (%) were used for the next step of analysis.

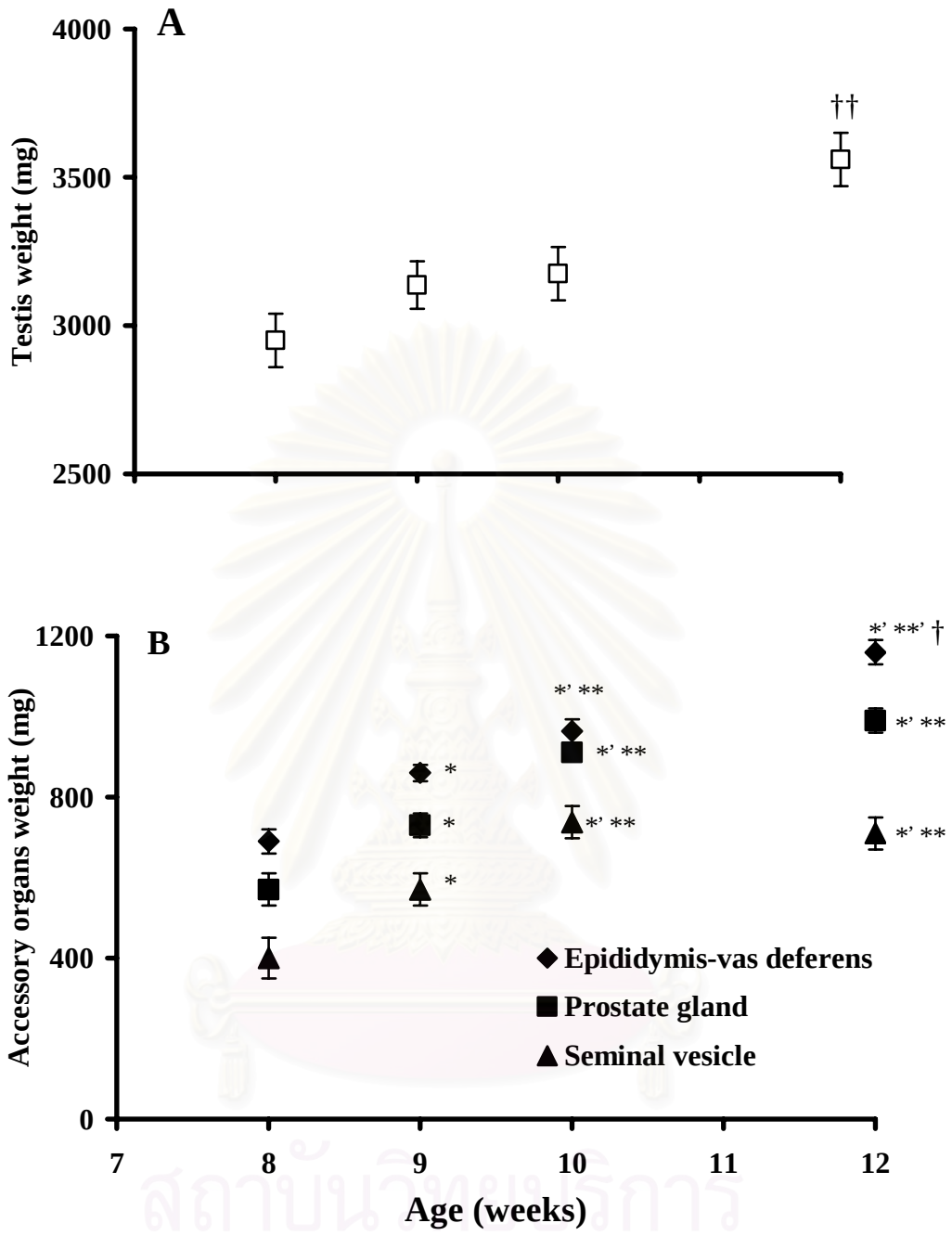

Figure 4.2 Weight gains of testes (A) and accessory sex organs (B) of normal male rats (Q0 group) from 8 to 12 weeks old. * = significant difference ($p < 0.05$) compared with 8 weeks. ** = significant difference ($p < 0.05$) compared with 9 weeks. † = significant difference ($p < 0.05$) compared with 10 weeks. †† = significant difference ($p < 0.05$) compared with 8, 9 and 10 weeks.

4.1.4 Effects of age on histological alterations of sex organs

There was no difference on histology of Q0 group in testis, epididymis, vas deferens, prostate gland and seminal vesicle (Figure 4.3, 4.4 and 4.5, respectively) between 8, 9, 10 and 12 weeks of age. The tubular area of seminiferous tubule, the tubular area and luminal area of epididymis (Table 4.1) did not significantly change with advancing age in Q0 group. However, the tubular thickness of epididymis at 9 weeks of age (or 7 days of study period) was significantly decreased compared with 8, 10 and 12 weeks of age (3 and 14 days of study period).

Table 4.1 Effects of age on histological alterations of sex organs during 8 weeks plus 3 days to 12 weeks old.

Parameters	Age (weeks)			
	8 weeks	**9 weeks**	**10 weeks**	**12 weeks**
Tubular area of seminiferous tubule	62.46 ± 1.89	61.49 ± 1.43	60.82 ± 1.31	60.63 ± 1.60
Tubular area of epididymis	105.32 ± 7.33	117.80 ± 10.20	113.66 ± 8.60	101.67 ± 5.70
Luminal area of epididymis	70.46 ± 4.17	73.26 ± 3.54	72.91 ± 7.06	71.03 ± 4.33
Tubular thickness of epididymis	33.80 ± 1.85	29.36 ± 1.90[1]	32.10 ± 1.43	33.23 ± 1.94

[1] = significant difference ($p<0.05$) compared with 8, 10 and 12 weeks of age

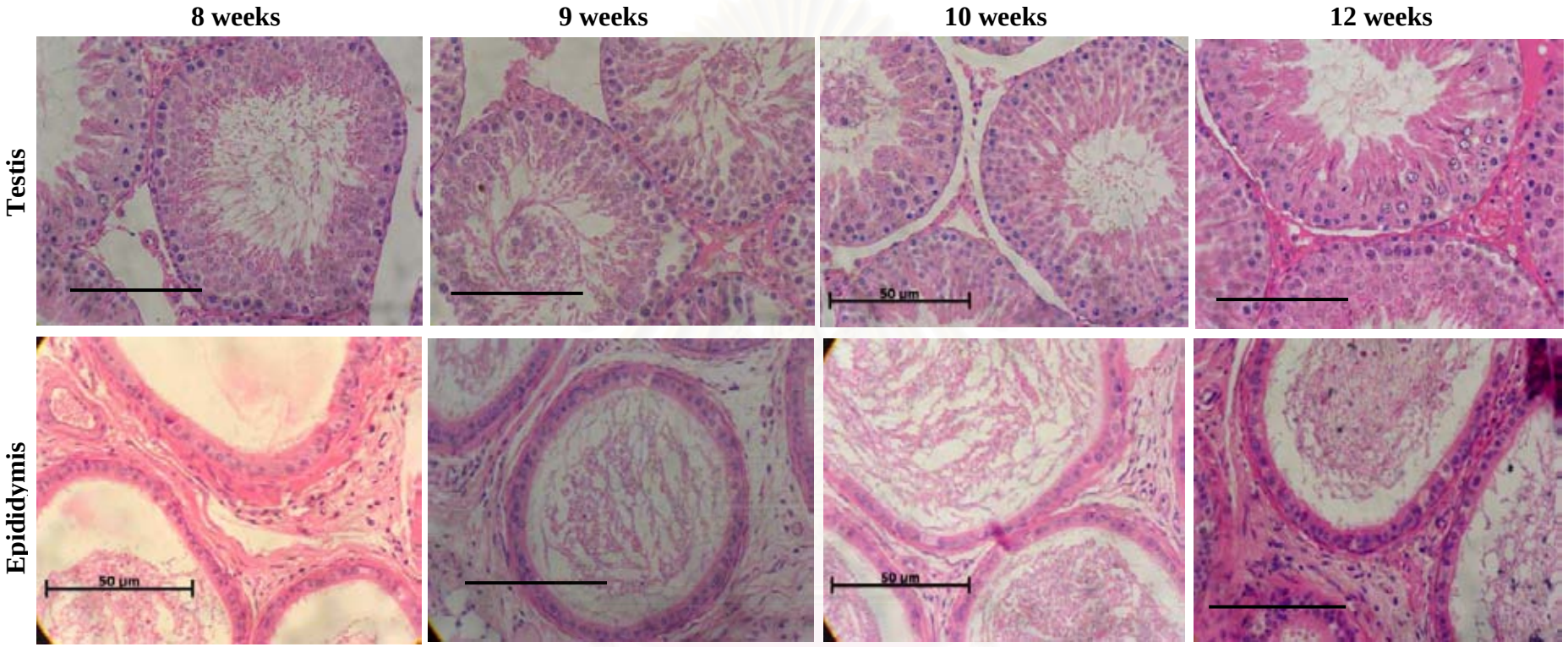

Figure 4.3 Structure of seminiferous tubules in testis (upper panel) and epididymis (lower panel) of intact control (Q0) rats at 8, 9, 10 and 12 weeks of age, respectively (H&E stained, 6 µm section). Scale bar =50 µm.

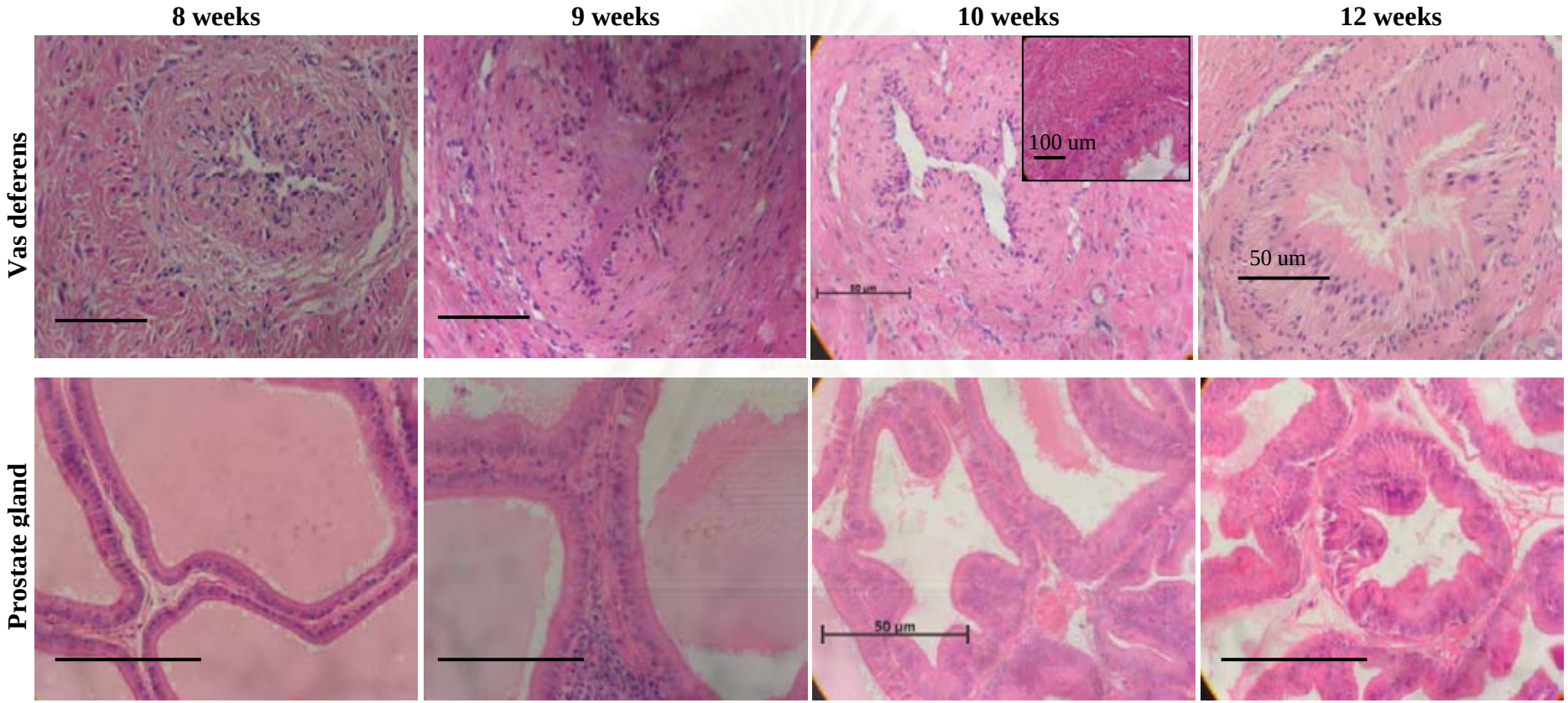

Figure 4.4 Structure of vas deferens (upper panel) and prostate gland (lower panel) of intact control (Q0) rats at 8, 9, 10 and 12 weeks of age, respectively (H&E stained, 6 µm section). Scale bar =50 µm.

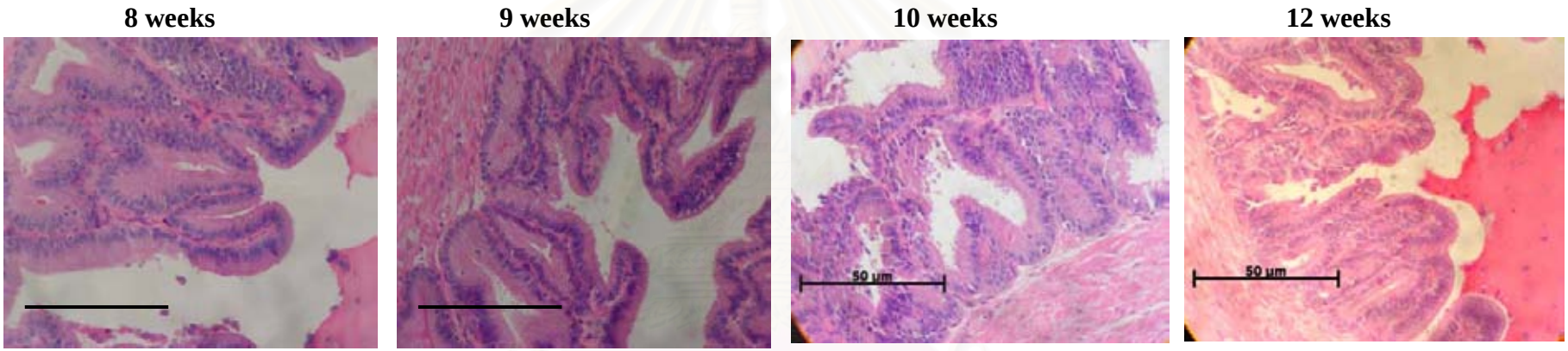

Figure 4.5 Structure of seminal vesicle of intact control (Q0) rats at 8, 9, 10 and 12 weeks of age, respectively (H&E stained, 6 µm section).

Scale bar =50 µm.

4.1.5 Effects of age on sperm quality

Only the sperm concentration showed a significant increase with advancing age of Q0 rats ($R^2 = 0.808$, $p<0.05$), whereas the sperm motility and sperm viability did not significantly increase (Figure 4.6).

The increase of sperm concentration started at 9 weeks of age (or 7 days of study period) until 12 weeks of age (or 28 days of study period). The increase seemed to reach a plateau at 10 weeks of age (or 14 days of study period).

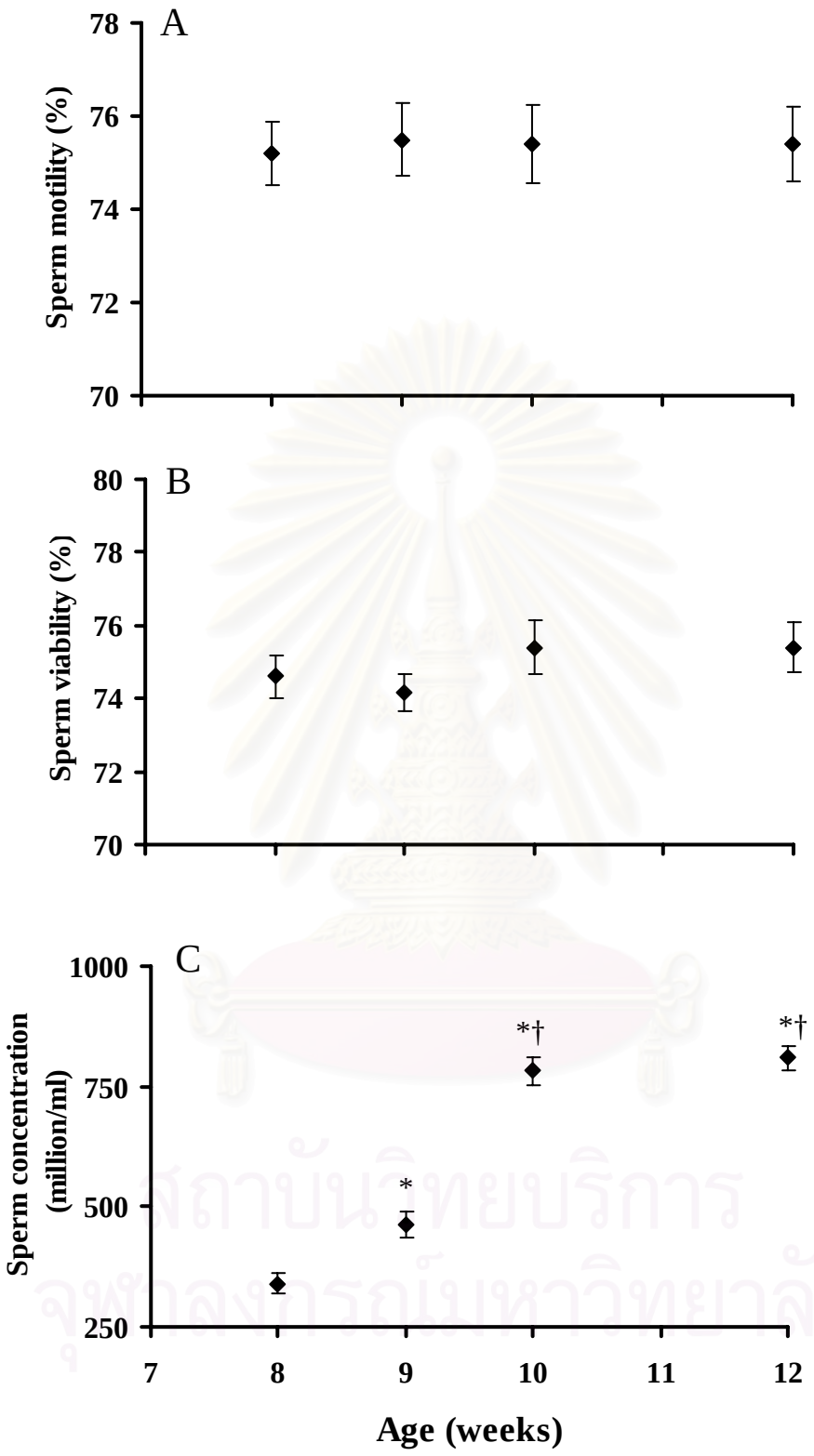

Figure 4.6 Sperm quality of male rats during 8-12 weeks old. * = significant difference ($p < 0.05$) compared with 8 weeks. † = significant difference ($p < 0.05$) compared with 9 weeks.

4.1.6 Effects of age on reproductive hormones and expression of StAR protein and P450scc

Serum FSH and testosterone levels did not significantly increase with advancing age of 8-12 weeks old rats (Figure 4.7). Serum LH levels were significantly increased only at 10 weeks of age (or 14 days of study period) compared with 8 weeks of age.

The expression of StAR protein and P450scc also did not significantly increase with advancing age (Figure 4.8). However, the expression of P450scc at 12 weeks of age was significantly higher than that of 9 weeks of age.

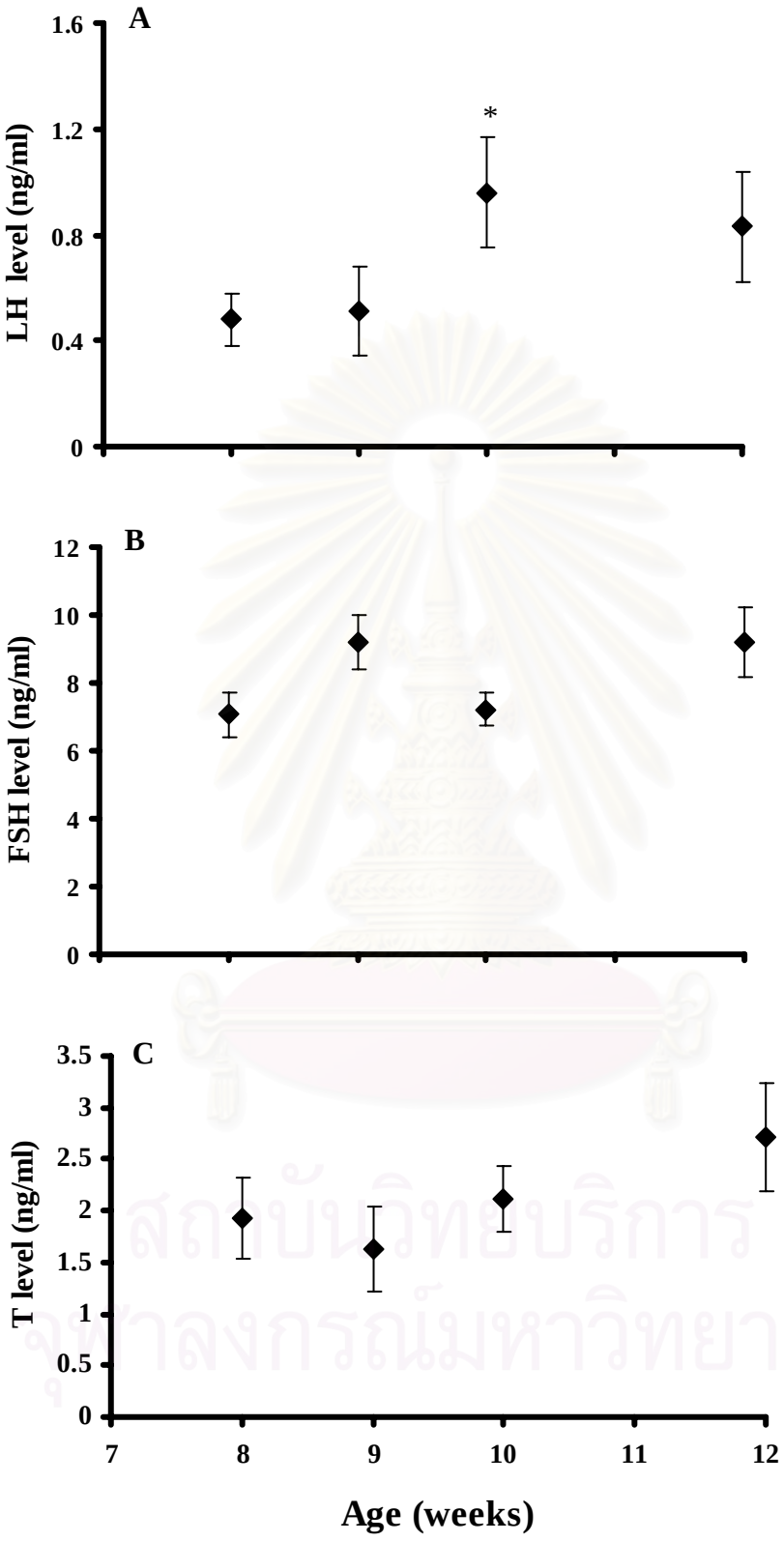

Figure 4.7 Gonadotropin and testosterone levels during 8-12 weeks old.

* = significant difference ($p < 0.05$) compared with 8 weeks.

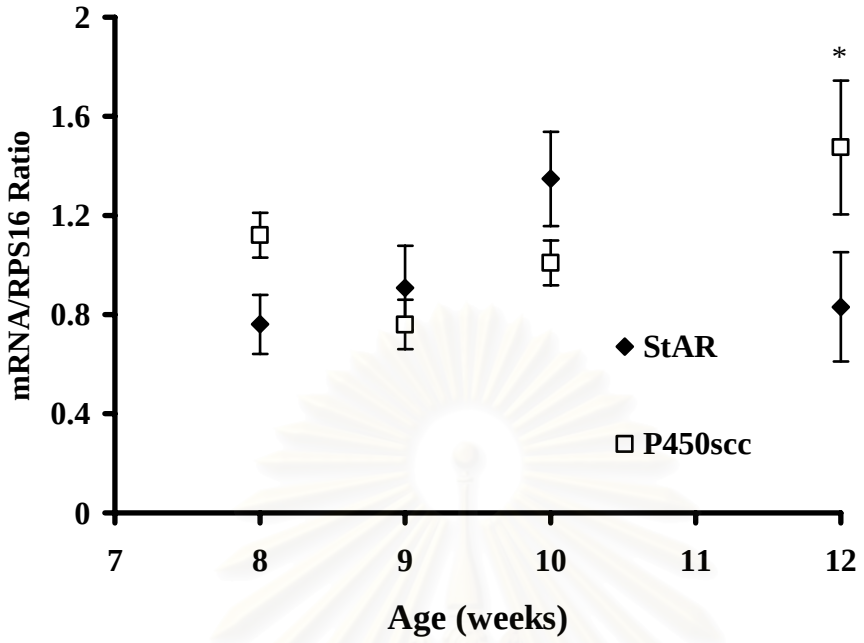

Figure 4.8 StAR protein and P450scc mRNA levels during 8-12 weeks old.

* = significant difference ($p < 0.05$) compared with 9 weeks.

4.2 Effects of quercetin on spermatogenesis in intact male rats

4.2.1 Effects of quercetin on body weight

There was no significant difference of rat body weights between Q0 and Q30, Q90 and Q270 groups at day 3, 7 and 14 and between Q0 and Q90 at day28 (Figure 4.9). With an increasing age from 8 to 12 weeks old, the body weights in Q30, Q90 and Q270 groups were significantly increased.

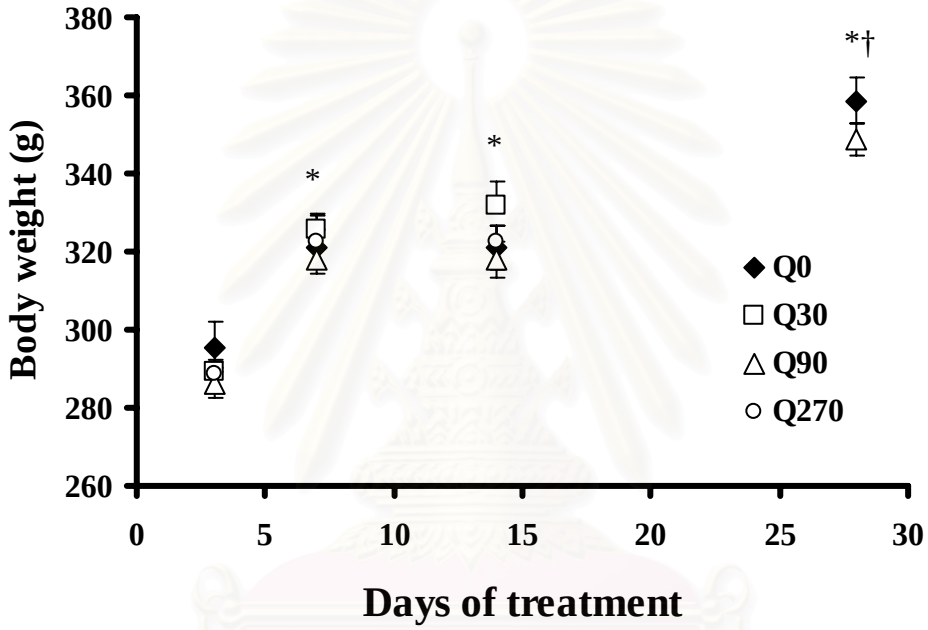

Figure 4.9 Body weights of intact male rats after subcutaneous injection of 0, 30, 90 or 270 mg/kgBW/day of quercetin (Q0, Q30, Q90 and Q270, respectively) for 3, 7, 14 and 28 days. * = significant difference ($p<0.05$) compared with day 3 of each treatments. † = significant difference ($p< 0.05$) compared with day 7 and 14 of each treatment.

4.2.2 Effects of quercetin on relative testis weights

Changes of relative organ weights were examined in two ways: dose dependence and time dependence (Table 4.2)

The increases of relative testis weights after quercetin treatment were depended on dose and time. The relative testis weights of Q270 group (after 7 and 14 days of treatment) and Q90 group (after 28 days of treatment) were significantly higher than those of the control group. There were no significant increase in the relative testis weights of Q30 and Q90 groups after 3, 7 and 14 days of treatment to that of Q0 group. However, when the relative testis weights were compared between day 3 and day 7, 14 and 28 in each dose, no significant difference was observed.

4.2.3 Effects of quercetin on relative weights of accessory sex organs

With regard to the quercetin doses, only the Q270 group (at 7 and 14 days) and Q90 group (at 28 days) exhibited a significant increase in relative epididymis-vas deferens weights over those of the control group (Table 4.2). The Q270 group (at day 14) and Q90 group (at day 28) showed a decrease of relative prostate gland and seminal vesicle weights compared with those of the control group (Table 4.3). No alterations in accessory sex organ weights were observed for the Q30 and Q90 treatment at day 3, 7 and 14 compared with those of the control group.

With regard to the duration of treatment (3-28 days), all four groups of quercetin-treated rats (Q0, Q30, Q90 and Q270) showed increases in relative weights of the epididymis-vas deferens. While the relative weights of prostate gland and seminal vesicle were increased at day 3-14 of treatment, but decreased at 28 days of treatment. The increases of relative sex organ weights were observed even in the Q0 group, which means that the weight gains of accessory sex organs were faster than the body weight gain.

Table 4.2 Relative weights of testis, epididymis-vas deferens of male rats after subcutaneous injections of 0, 30, 90 or 270 mg/kg BW/day of quercetin for 3, 7, 14 and 28 days.

Quercetin	Testis				Epididymis-vas deferens			
	3 days	**7 days**	**14 days**	**28 days**	**3 days**	**7 days**	**14 days**	**28 days**
Q0	0.99±0.06	0.98±0.11	0.99±0.05	0.99 ± 0.07	0.23±0.03	0.27±0.03 [1]	0.30±0.02 [1,2]	0.32 ± 0.03 [1,2,3]
Q30	1.01±0.08	1.02±0.07	1.00±0.03		0.24±0.02	0.28±0.02 [1]	0.30±0.02 [1,2]	
Q90	1.03±0.07	1.03±0.06	1.01±0.06	1.06 ± 0.08 [a]	0.24±0.03	0.26±0.03 [1]	0.30±0.01 [1,2]	0.35 ± 0.02 [1,2,3 a]
Q270	1.05±0.06	1.06±0.07 [a]	1.07±0.07 [a]		0.25±0.02	0.29±0.02 [1 ac]	0.33±0.03 [1,2 abc]	

[a, b] and [c] = significant difference ($p < 0.05$) compared with Q0, Q30 and Q90, respectively

[1, 2, 3] = significant difference ($p < 0.05$) compared with 3, 7 and 14days, respectively

Table 4.3 Relative weights of prostate gland and seminal vesicle of male rats after subcutaneous injections of 0, 30, 90 or 270 mg/kg BW/day of quercetin for 3, 7, 14 and 28 days.

Quercetin	Prostate gland				Seminal vesicle			
	3 days	7 days	14 days	28 days	3 days	7 days	14 days	28 days
Q0	0.19±0.03	0.23±0.04 [1]	0.28±0.02 [1,2]	0.28 ± 0.03 [1,2]	0.13±0.05	0.18±0.04 [1]	0.23±0.04 [1,2]	0.20 ± 0.05 [1,2,3]
Q30	0.19±0.03	0.22±0.03 [1]	0.24±0.02 [1,2]		0.15±0.04	0.17±0.03 [1]	0.20±0.05 [1,2]	
Q90	0.21±0.03	0.20±0.03	0.26±0.05 [1,2]	0.23 ± 0.04 [1,2,3 a]	0.14±0.06	0.16±0.03 [1]	0.22±0.05 [1,2]	0.13 ± 0.04 [2,3 a]
Q270	0.19±0.02	0.23±0.03 [1]	0.23±0.04 [1 ac]		0.14±0.02	0.16±0.02 [1]	0.15±0.03 [abc]	

[a, b] and [c] = significant difference ($p < 0.05$) compared with Q0, Q30 and Q90, respectively

[1, 2, 3] = significant difference ($p < 0.05$) compared with 3, 7 and 14days, respectively

4.2.4 Effects of quercetin on histological alterations of testis

The stratified epithelium of the seminiferous tubules contained different stages of developing sperm cells in the control group (Q0 group) (Figure 4.10). There was no difference in the structure of the seminiferous tubules in any treatment groups between day 3 and day 7. However, at day 14 and 28 of Q90 group and day 14 of Q270 group, the retention of spermatozoa in seminiferous tubules was higher than those in the Q0 and Q30 groups. Therefore, only the histological structure of seminiferous tubules in testis at day 14 and day 28 are shown.

The tubular area of seminiferous tubules was increased in time and dose dependent manner. Only the Q270 group showed a significantly higher value than the control group and the lower dosage groups (Q30 and Q90) at day 14 (Table 4.4). The tubular area of seminiferous tubules in Q90 group was also significantly higher than that of the control group after 28 days of treatment. Histological examination of the testis did not reveal any evidence of degeneration of germ cells in any dose groups.

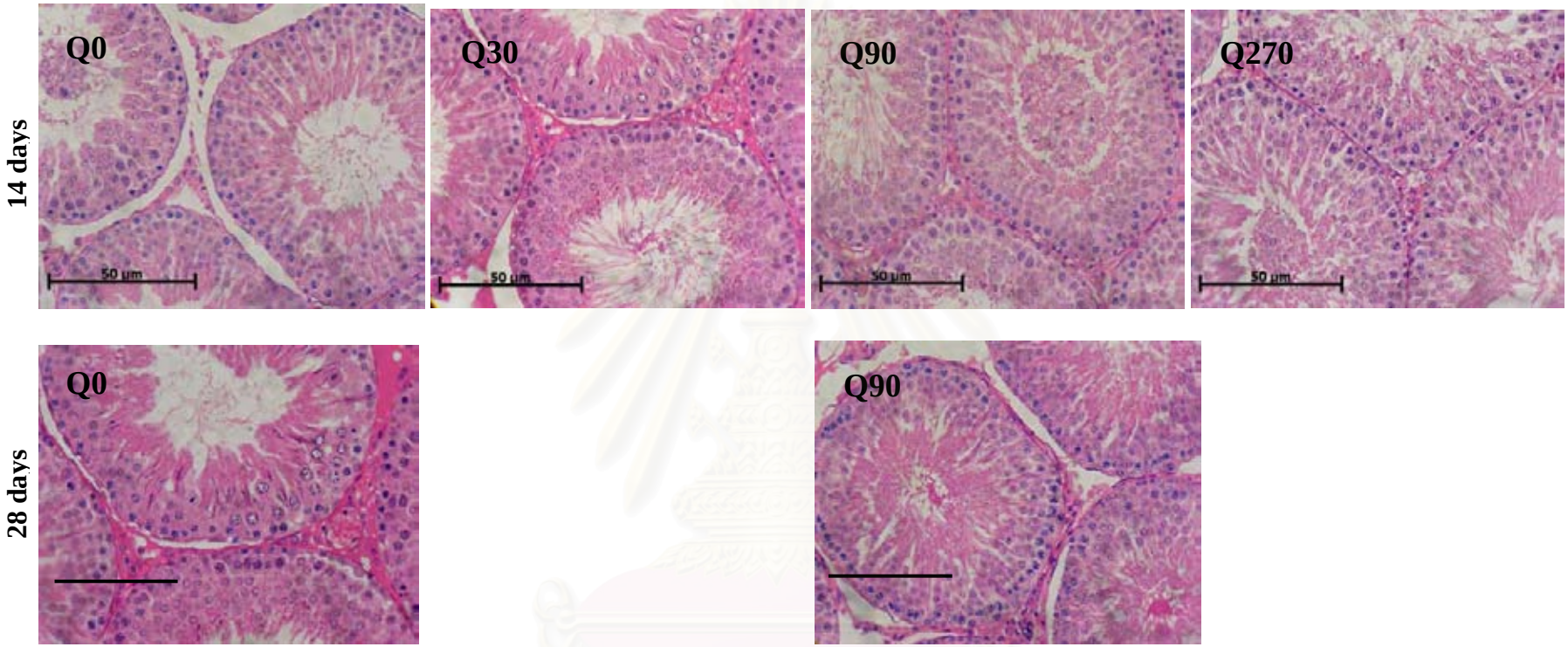

Figure 4.10 Structure of seminiferous tubules in testis of intact male rats after subcutaneous injections of quercetin at dose of 0, 30, 90 or 270 mg/kgBW/day for 14 days (upper panel) and 28 days (lower panel) (H&E stained, 6 µm section). Scale bar = 50 µm.

Table 4.4 Tubular area of seminiferous tubule after subcutaneous quercetin injection at 0, 30, 90 or 270 mg/kgBW/day for 3, 7, 14 days and 28 days.

Quercetin	Tubular area of seminiferous tubule ($x 10^3 \mu m^2$)			
	3 days	**7 days**	**14 days**	**28 days**
Q0	62.46 ± 1.89	61.49 ± 1.43	60.82 ± 1.31	60.63 ± 1.60
Q30	61.34 ± 1.31	63.50 ± 1.67	61.65 ± 1.06	
Q90	60.67 ± 1.56	63.61 ± 1.45	63.33 ± 1.32	63.63 ± 1.23 [1 a]
Q270	62.80 ± 1.78	63.54 ± 1.34	66.61 ± 1.25 [1 abc]	

[a, b] and [c] = significant difference ($p< 0.05$) compared with Q0, Q30 and Q90, respectively

[1] = significant difference ($p< 0.05$) compared with 3 days

4.2.5 Effects of quercetin on histological alterations of accessory sex organs

The accessory sex organs (epididymis-vas deferens, prostate gland and seminal vesicle) did not show any difference in histological structure between the control (Q0) and treatment groups (Q30, Q90 and Q270) at day 3.

The epididymis of the control group (Q0 group) consisted of numerous tubes filled with spermatozoa and fluid (Figure 4.11). The epididymal tubes were lined with a very tall pseudostratified stereociliated cuboidal epithelium. Most epithelial cells (or principal cells) have long stereocilia. In Q90 group at day 14 and 28 and Q270 group at day 14, the numbers of epididymal tubes containing spermatozoa and fluid were slightly higher than that of the Q0 group, and the epithelia were lined with pseudostratified cuboidal cells (Figure 4.11). Principal cells of the epithelium in Q90 group at day 14 were vacuolarized and had more stereocilia than the Q0 group. However, the principal cells of the epithelium in Q90 group at day 28 had no vacuole and less stereocilia compared with Q90 at day 14.

Changes of the tubular and luminal areas of epididymis depended on dosage and time (Table 4.5). Compared with the day 3, only Q270 group showed a significant increase in tubular area of epididymis at day 7 and day 14.

Compared with Q0 group, the tubular area and luminal area of epididymis of Q90 (at day 14 and 28) and Q270 (at day 7 and 14) showed significant difference increases in size. The luminal area of epididymis of Q90 at day 7 was also higher than that of Q0 group.

The increase in tubular thickness of epididymis was depended on the increase in ratio of tubular area to luminal area. Compared with Q0 group, the tubular thickness of epididymis increased only at day 7 of Q90 and Q270. Compared with

day 3, the tubular thickness at day 7 of Q0, at day 28 of Q90 and at day 14 of Q270 were lower, whereas the tubular thickness at day 7 of Q270 group was higher.

Table 4.5 Tubular area, luminal area and tubular thickness of epididymis after subcutaneous quercetin injection at 0, 30, 90 or 270 mg/kgBW/day for 3, 7 and 14 days. Tubular thickness (μm^2) = Tubular area – luminal area

Quercetin	Tubular area (x 10^3 μm^2)			
	3 days	7 days	14 days	28 days
Q0	105.32 ± 7.33	117.80 ± 10.20	113.66 ± 8.60	101.67 ± 5.70
Q30	109.65 ± 5.32	121.81 ± 7.56	115.78 ± 3.70	
Q90	120.20 ± 10.12	130.12 ± 8.02	131.27 ± 5.91 [ab]	138.18 ± 9.38 [a]
Q270	124.30 ± 13.70	178.35 ± 17.10 [1abc]	182.56 ± 20.80 [1 abc]	
Quercetin	**Luminal area (x 10^3 μm^2)**			
	3 days	**7 days**	**14 days**	**28 days**
Q0	70.46 ± 4.17	73.26 ± 3.54	72.91 ± 7.06	71.03 ± 4.33
Q30	71.53 ± 5.92	82.44 ± 8.41	86.14 ± 6.27 [1]	
Q90	80.25 ± 8.38	88.15 ± 8.21 [a]	94.54 ± 6.52 [1 a]	106.39 ± 8.23 [1 a]
Q270	83.35 ± 10.20	90.77 ± 6.82 [a]	103.68 ± 5.21 [1,2 ab]	
Quercetin	**Tubular thickness (μm^2)**			
	3 days	**7 days**	**14 days**	**28 days**
Q0	33.80 ± 1.85	29.36 ± 1.90 [1]	32.10 ± 1.43 [2]	33.23 ± 1.94 [2]
Q30	33.19 ± 1.32	32.35 ± 1.57	30.75 ± 1.95	
Q90	39.27 ± 3.67	45.14 ± 3.85 [ab]	32.27 ± 1.28	31.79 ± 1.66 [1,2]
Q270	36.85 ± 1.88	43.42 ± 3.22 [1 ab]	31.09 ± 1.14 [1,2]	

[a] and [b] = significant difference ($p < 0.05$) compared with Q0 and Q30, respectively

[1] and [2] = significant difference ($p < 0.05$) compared with 3 and 7 days, respectively

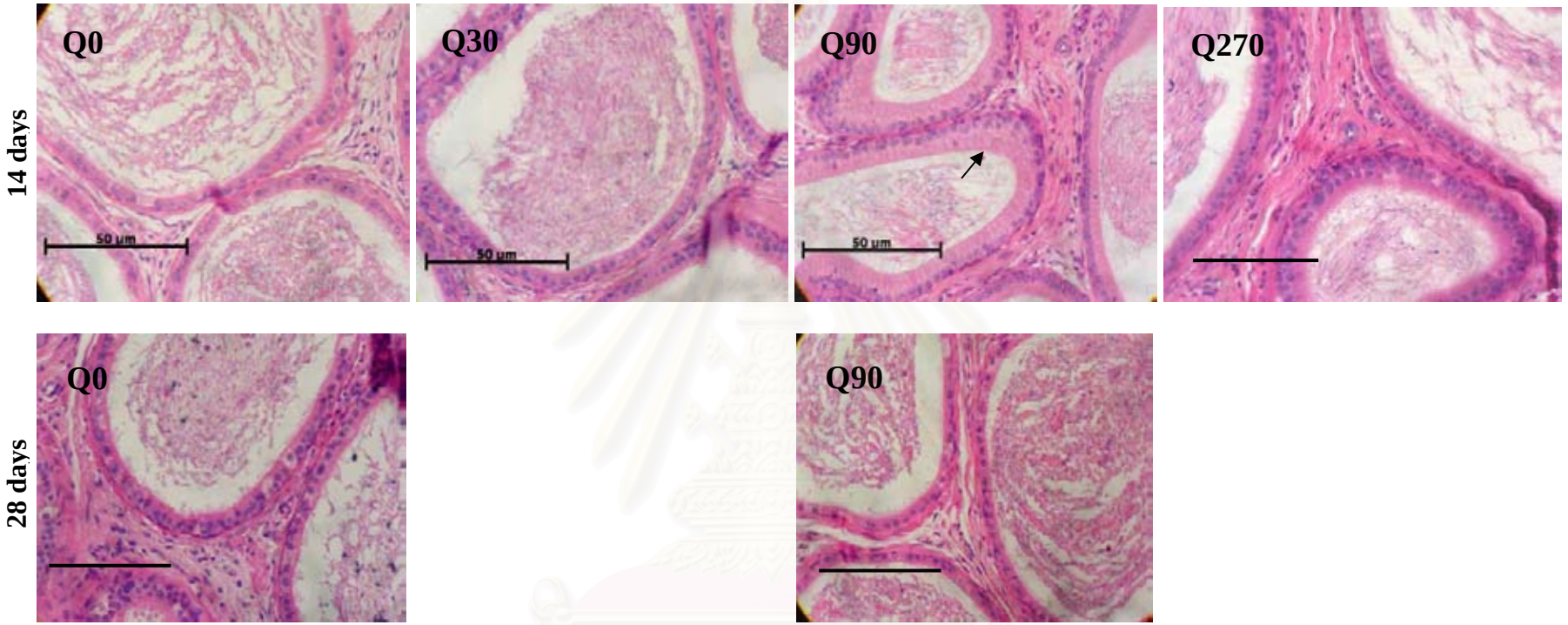

Figure 4.11 Structure of epididymis of intact male rats after subcutaneous injections of quercetin at dose of 0, 30, 90 or 270 mg/kgBW/day for 14 days (upper panel) and 28 days (lower panel) (H&E stained, 6 µm section). Scale bar = 50 µm. Black arrow indicates long stereocilia of epithelium cells in the Q90 group (B3).

The mucosa of the vas deferens of the control group formed longitudinal folds and lined with pseudostratified columnar epithelial cells with long stereocilia (Figure 4.12). There were no differences in the vas deferens between control and treatment groups, regardless of either the quercetin dose or the duration of treatment

The prostate gland of the control group (Q0 group) contained many tubuloalveolar glands or secretory alveoli (Figure 4.13). The secretory alveoli were lined with a layer of tall columnar epithelial cells with a high cytoplasm/nucleus ratio. The epithelial lining also had irregular shape because the mucosa had papillary projections into the lumen of the gland. The lumen was filled with secretory fluid. The prostate gland observed in the Q30 group was similar to that of the Q0 group. The prostate lumen in the Q90 and 270 groups at day 14 and Q90 at day 28 were highly dilated and the number of tubes found in each section was also decreased. The luminal epithelial cells in the Q90 and Q270 groups at both day 14 and 28 showed a marked reduction in cytoplasm and thickness of mucosa compared with the control group.

The seminal vesicle of the control group (Q0 group) was complex with glandular structure, and the lumen was highly irregular and recessed with honeycomb-like features (Figure 4.14). The mucosa of the seminal vesicle of Q0 group exhibited thick, branched, and anastomossing folds that were less distinct in the quercetin treated group. The epithelia had a varied appearance of columnar or pseudostratified columnar. In agreement with the decrease seminal vesicle weights, rats treated with Q270 for 14 days showed markedly dilated lumens and markedly reduced branching of the mucosa of seminal vesicles in comparison with those of Q0, Q30 and Q90 groups. In addition, the mucosal epithelia of Q270 rats had either cuboidal or

squamous cells. The Structure of seminal vesicle of Q90 at day 28 was similar to that of day 14.

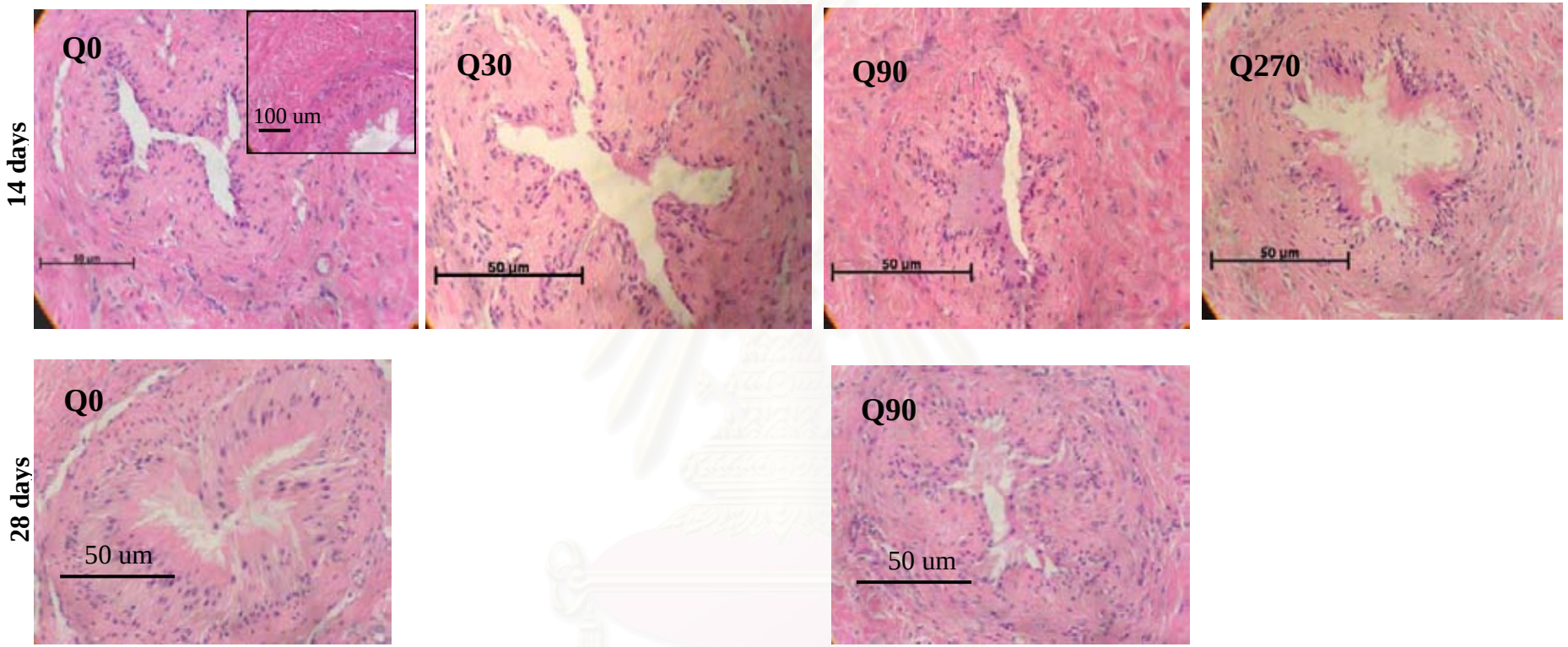

Figure 4.12 Structure of vas deferens of intact male rats after subcutaneous injections of quercetin at dose of 0, 30, 90 or 270 mg/kgBW/day for 14 days (upper panel) and 28 days (lower panel) (H&E stained, 6 µm section). Scale bar = 50 µm.

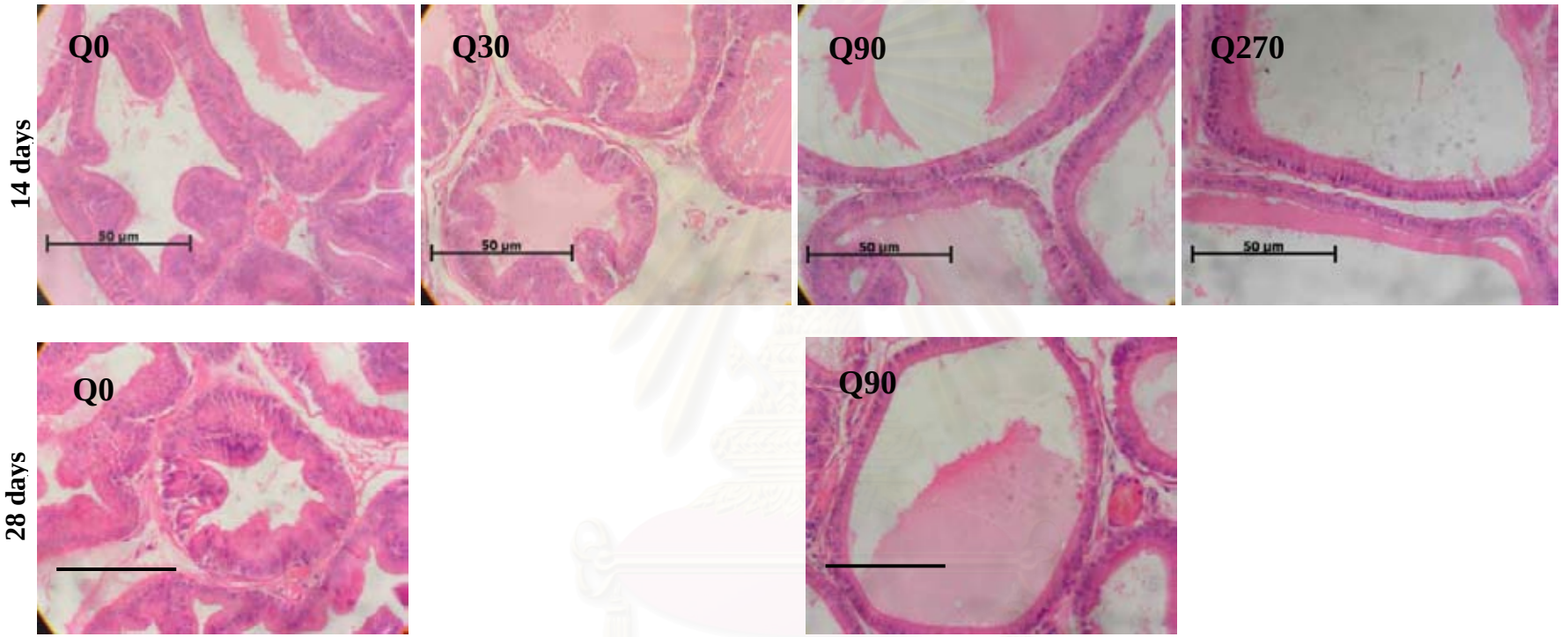

Figure 4.13 Structure of prostate gland of intact male rats after subcutaneous injections of quercetin at dose of 0, 30, 90 or 270 mg/kgBW/day for 14 days (upper panel) and 28 days (lower panel) (H&E stained, 6 µm section). Scale bar = 50 µm.

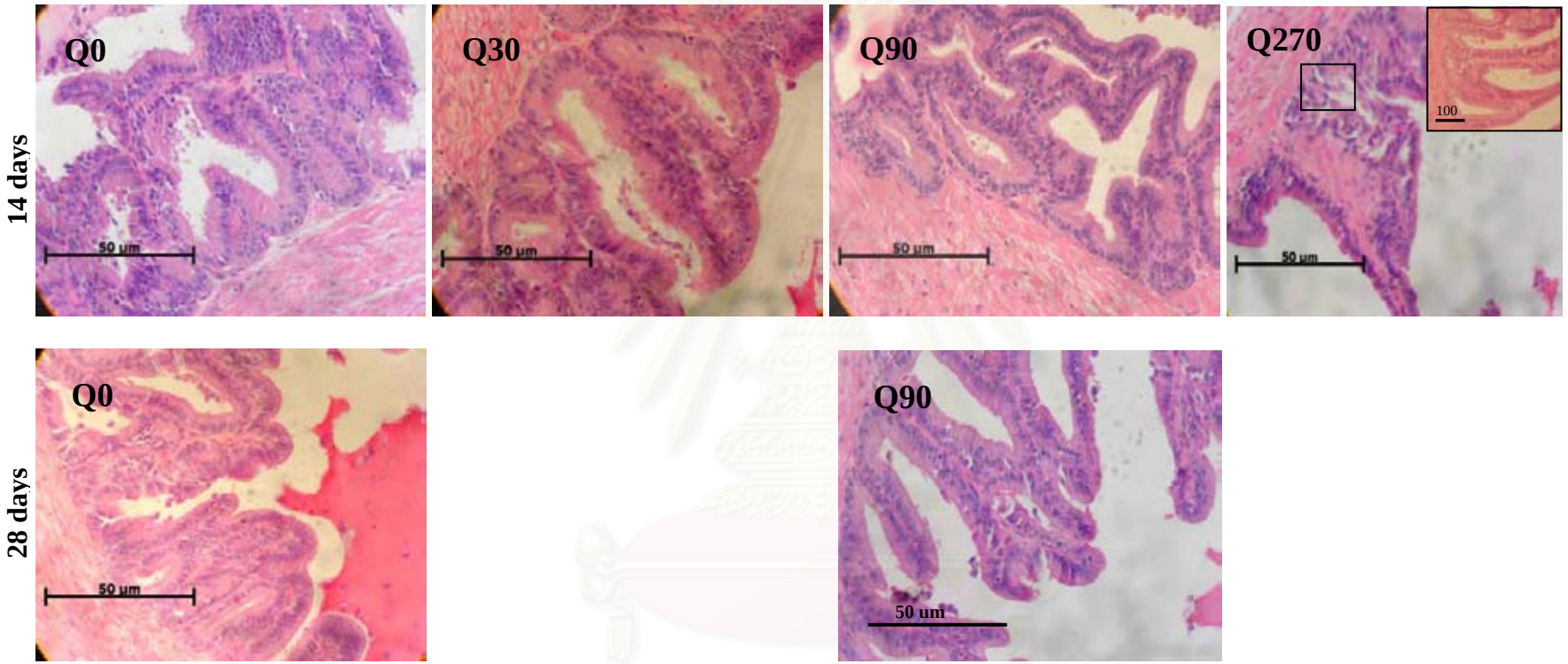

Figure 4.14 Structure of seminal vesicle of intact male rats after subcutaneous injections of quercetin at dose of 0, 30, 90 or 270 mg/kgBW/day for 14 days (upper panel) and 28 days (lower panel) (H&E stained, 6 µm section). Scale bar = 50 µm.

4.2.5 Effects of quercetin on sperm quality

There was no difference in sperm quality (sperm motility, sperm viability and sperm concentration) after all three doses of quercetin treatment (Q30, Q90 and Q270) for 3 days, compared with the control group (Q0) (Figure 4.15). However, the increases in sperm quality depended on both of dose and duration of quercetin treatment afterward. With regard to the dose, the motility, viability and concentration of sperm in the Q90 and Q270 groups were higher than that of the control group when the duration of treatment was prolonged to 7 and 14 days. However, only the sperm concentration and sperm motility were increased at day 28 of Q90 group compared with the control group.

Compared with Q0 group, the sperm motility of Q30 (at day 14), Q90 and Q270 (at day 7 and 14) showed significant increase. The sperm viability of Q30 (at day 14), Q90 (at day 14) and Q270 (at day 7 and 14) showed significant increase compared with that of control group. The sperm concentration of all doses of quercetin (at day 14) and Q90 (at day 28) were significantly higher compared with the control group.

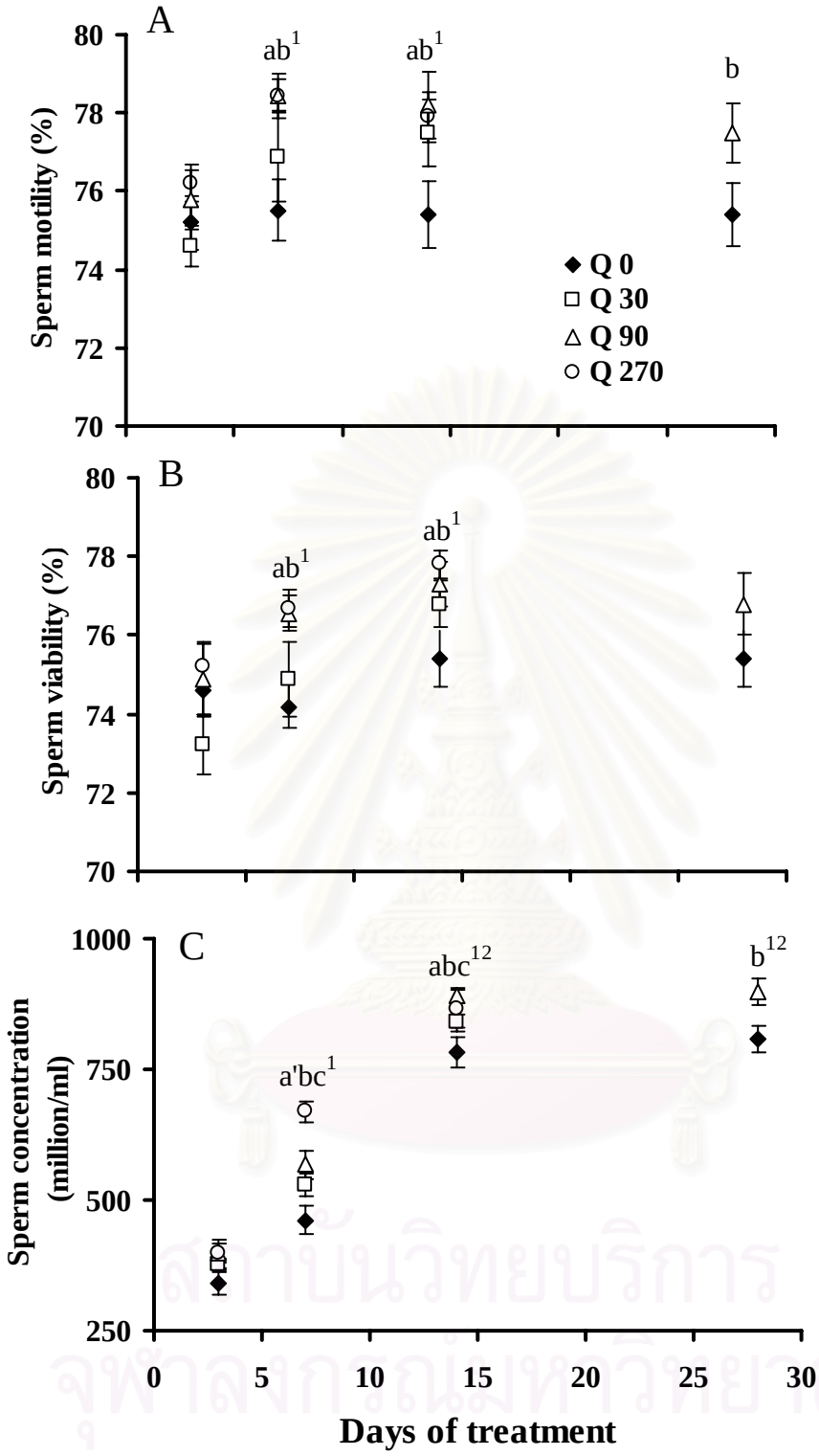

Figure 4.15 Sperm motility (A), viability (B) and concentration (C) of male rats after subcutaneous injections of 0, 30, 90 or 270 mg/kgBW/day of quercetin for 3, 7, 14 and 28 days. [a'] = significant difference ($p < 0.05$) between Q270 and Q0, Q30 and Q90. [a, b] and [c] = significant difference ($p < 0.05$) between Q0 and Q270, Q90 and Q30, respectively. [1] = significant difference ($p < 0.05$) compared with day 3 of treatment. [2] = significant difference ($p < 0.05$) compared with day 7 of treatment.

4.3 Effects of quercetin on steroidogenesis and its mechanisms

4.3.1 Effects of quercetin on serum gonadotropin and testosterone levels

Serum LH levels were found to decrease in relation to dose and duration of treatment (Figure 4.16A). With regard to the dose, serum LH levels in Q30 and Q90 groups at day 3 were not significantly different from Q0. However, when the dose was increased to 270 mg/kgBW/day, the serum LH levels were significantly decreased compared with that of the Q0 group. Serum LH levels of Q270 group were lower than the Q0 group throughout the 14 days treatment. Serum LH levels of the Q30 and Q90 were significantly lower than the Q0 group when the duration of treatment was increased to7 and 14 days. The result was more pronounced in the Q90 group whose was markedly decreased serum LH level when the duration of treatment was 28 days.

In contrast to changes of serum LH levels, there were no reduction in serum FSH levels was found in any doses after 3 and 7 days of treatment (Figure 4.16B). It is necessary to note that serum FSH levels at day 3 of the Q30 group was significantly higher than other 3 groups. At day 7, serum FSH levels decreased in every doses of quercetin treatment. However, at day 14, only FSH levels of the Q270 group was significantly lower than that of the Q0 group. Serum FSH levels of all 4 groups at day 14 were also lower than at day 7 and day 28. At day 28, FSH levels of Q90 group was found to decrease significantly compared with that of the control group.

Changes of serum testosterone levels did not follow the pattern of changes of serum LH and FSH levels (Figure 4.16C). In general, testosterone levels of the control group tended to be higher than the treated group. Serum testosterone levels were found to decrease significantly in Q90 and Q270 group compared to that

of the control at day 3 of treatment. Serum testosterone levels of the Q270 group was also significantly lower than that of the control group at day 14 of treatment. Moreover, serum testosterone levels of Q0 group were very fluctuated throughout 28 days of study period and it tended to be increased with advancing age.

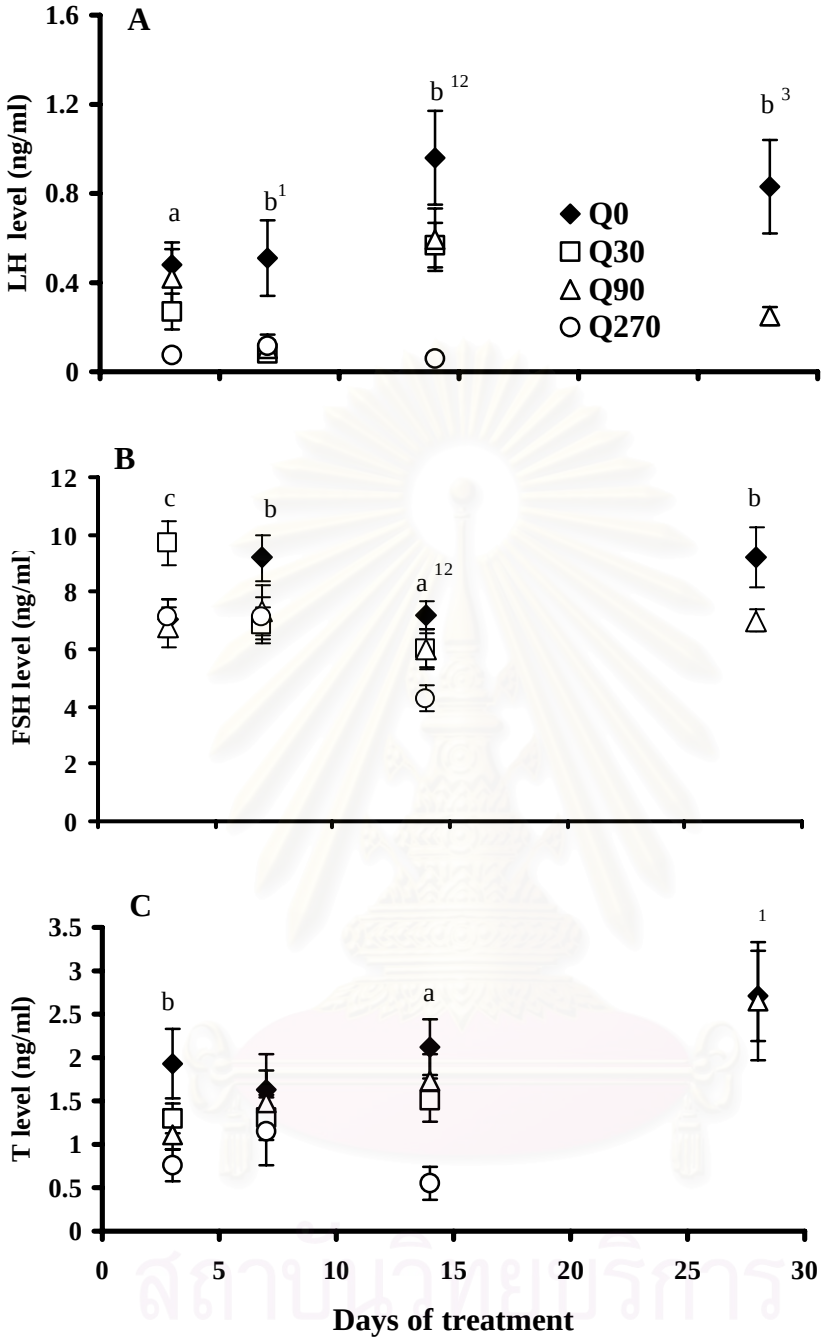

Figure 4.16 Changes of serum hormone levels in normal rats after subcutaneous injections of 0, 30, 90 or 270 mg/kgBW/day of quercetin for 3, 7, 14 and 28 days. a = significant difference ($p < 0.05$) between Q270 and Q0, Q30 and Q90. b = significant difference ($p < 0.05$) between Q0 and Q30, Q90 and Q270. c = significant difference ($p < 0.05$) between Q30 and Q0, Q90 and Q270. [1] = significant difference ($p < 0.05$) compared with day 3 of treatment. [2] = significant difference ($p < 0.05$) compared with day 7 of treatment. [3] = significant difference ($p < 0.05$) compared with day 14 of treatment.

4.3.2 Effects of quercetin on the expression of StAR protein and P450scc

4.3.2.1 StAR protein mRNA

The expression of StAR protein of Leydig cell was interpreted from the detection of mRNA levels which were reversely transcribed into cDNA levels by the RT-PCR technique. The PCR product of StAR protein was normalized with RPS16 levels. Comparison of the StAR protein mRNA levels showed no significant difference between any doses of quercetin after treatment for 3-28 days (Figure 4.17). However, StAR protein mRNA levels in each group tend to increase during 7 to 14 days treatment.

Compared with day 3, StAR protein mRNA levels of Q30, Q90 and Q270 groups at day 7 and day 14 were significantly increased. Compared with day 7, the mRNA level was also increased in Q30 and Q270 groups at day 14.

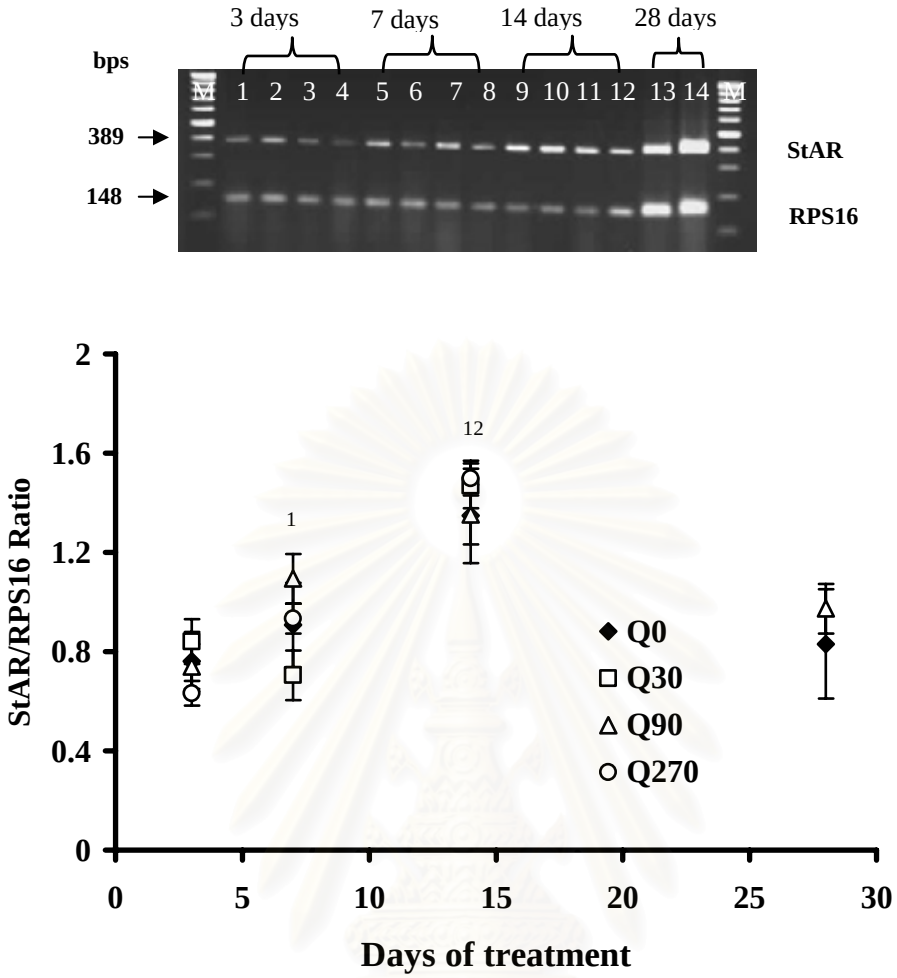

Figure 4.17 Changes of StAR protein mRNA in normal rats after subcutaneous injections of 0, 30, 90 or 270 mg/kgBW/day of quercetin for 3, 7, 14 and 28 days. [1] = significant difference ($p < 0.05$) compared with day 3 of treatment. [2] = significant difference ($p < 0.05$) compared with day 7 of treatment. M = 100 bp Marker**.** Lane 1, 5, 9 and 13 = Q0. Lane 2, 6, and 10 = Q30. Lane 3, 7, 11 and 14 = Q90. Lane 4, 8, and 12 = Q270.

4.3.2.2 P450scc mRNA

The expression of P450scc of Leydig cell was also analyzed in the same way as did for the StAR protein. Comparing the levels of P450scc mRNA, it was found that there were no significant difference in P450scc mRNA levels between any doses of quercetin after treatment for 3-14 days. The significant reduction of P450scc mRNA levels was observed only after 28 days of Q90 treatment (Figure 4.18).

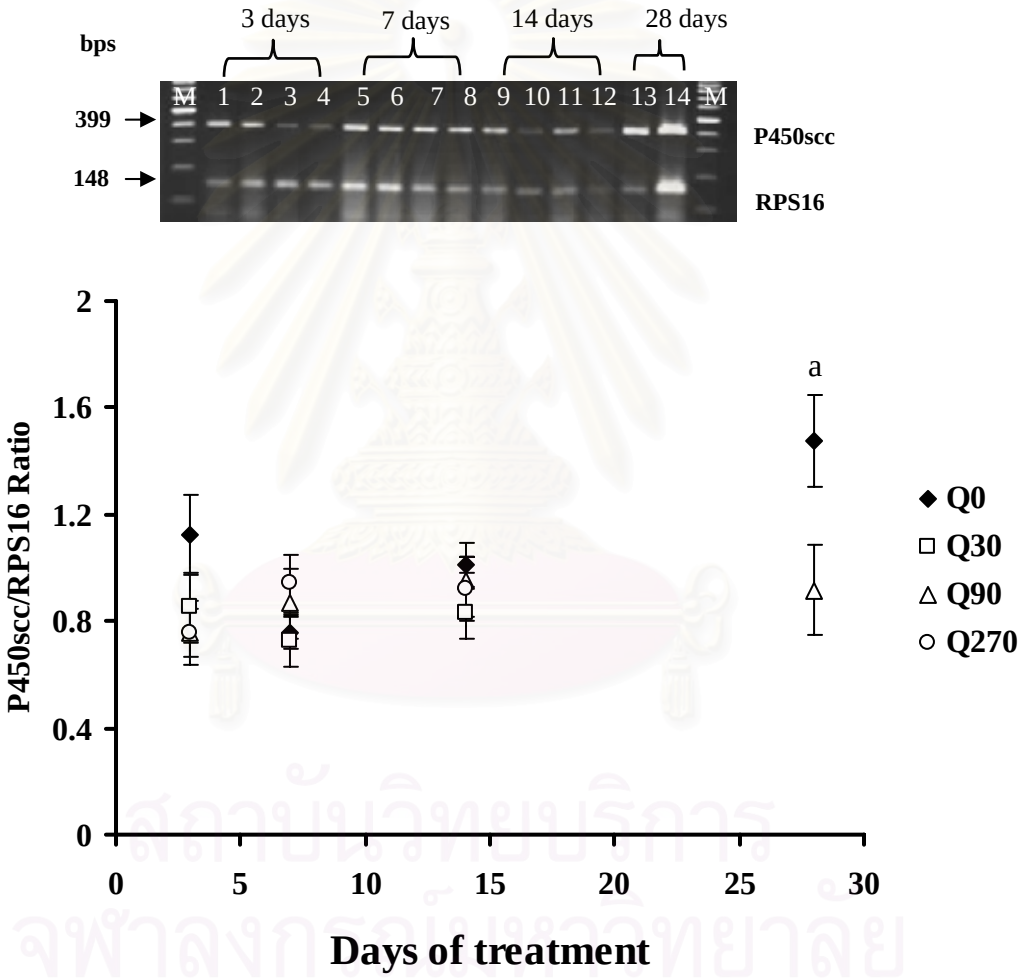

Figure 4.18 Changes of P450scc mRNA in normal rats after subcutaneous injections of 0, 30, 90 or 270 mg/kgBW/day of quercetin for 3, 7, 14 and 28 days. M = 100 bp Marker**.** Lane 1, 5, 9 and 13 = Q0. Lane 2, 6, and 10 = Q30. Lane 3, 7, 11 and 14 = Q90. Lane 4, 8, and 12 = Q270. [a] = significant difference ($p<0.05$) compared with Q0.

Table 4.6 Relationship between age and quercetin treatment on accessory sex organs weights and histology, hormone levels, StAR protein and P450scc expression in intact male rats.

Parameters	Correlation coefficient (R^2)		Summarizing effects of age (A) and quercetin (Q) on parameters
	Age effect	Quercetin effect	
Body weight	0.7843 (+)	0.0335 (0)	A
Testis weight	0.0009 (0)	0.7505 (++)	Q
Epididymis-vas deferens weight	0.7878 (++)	0.0179 (0)	A
Prostate gland weight	0.5080 (+)	0.0613 (-)	A ≠ Q
Seminal vesicle weight	0.1080(+)	0.1439 (-)	A ≠ Q
Tubular area of seminiferous tubules	0.0018 (0)	0.4316 (++)	Q
Tubular area of epididymis	0.0012 (0)	0.6725 (+)	Q
Luminal area of epididymis	0.1307 (+)	0.3669 (+)	Q > A
Thickness area of epididymis	0.1329 (-)	0.1738 (+)	A ≠ Q
Sperm motility	0.0358 (+)	0.2922 (++)	Q > A
Sperm viability	0.2216 (+)	0.2473 (+)	Q > A
Sperm concentration	0.6788 (++)	0.0093 (0)	A
LH levels	0.1500 (+)	0.4360 (--)	A ≠ Q
FSH levels	0.0021 (0)	0.2536 (-)	Q
Testosterone levels	0.4971 (++)	0.4321 (--)	A ≠ Q
StAR mRNA	0.1210 (+)	0.0038 (0)	A
P450scc mRNA	0.3450 (+)	0.0629 (0)	A

0 = no significant difference ($p>0.05$)

+ and ++ = significantly increased at $p<0.05$ and 0.01, respectively

- and -- = significantly decreased at $p<0.05$ and 0.01, respectively

4.4 Effects of quercetin on fertility in male rats

In this study, male rat of each group was kept with two female rats for one complete estrous cycle (or 5 days) at day 10-14 in the 14-day treatment schedule and at day 24-28 of the 28-day treatment schedule. Therefore, the mating between male and female rats should occur on choice when the female rats were on the estrous stage of estrous cycle or during the endogenous estrogen levels were high. Although the mating was on choice, mean of the mating times between Q0, Q90 and Q270 in the 14-day treatment schedule and between Q0 and Q90 in the 28-day treatment schedule were not significantly different (Table 4.7). Most of male rats mated on day 12 and day 26 in the 14-day and 28-day treatment, respectively.

The percentage of male with sperm positive female and male fertility index in the Q270 group were significantly lower than other 2 groups (Q0 and Q90). Thus, the number of pregnant females was lower in the Q270 group. The percentage of male with sperm positive female in the Q90 group at day 28 was significantly higher than that of the Q90 group at day 14. The number of fetus per litter was also significantly decreased in the Q270 group whereas the percentage of resorptions was significantly increased. Comparison between the 14-day and the 28-day treatment of the control group (Q0) showed no significant difference in any parameters.

Table 4.7 Fertility testing of male rats treated with quercetin at dose of 0, 90 or 270 mg/KgBW for 14 and 28 days.

Groups	Mean of mating time (days)	% of males with sperm positive females [1]	% of Male fertility index [2]	No. of males pregnating			No. of pregnant females [3] (%)	No. of fetus per litter	% Reabsorption [4]
14 days				0	1	2			
Q0	12.70 ± 1.14 (10-14)	80 (8/10)	100 (10/10)	0	4	6	16 (80)	7.10	5.33 (8/150)
Q90	12.00 ± 1.00 (10-13)	80 (8/10)	100 (10/10)	0	3	7	17 (85)	7.90	5.34 (9/167)
Q270	12.33 ± 0.58 (10-14)	60 (6/10) [a]	80 (8/10) [a]	2	3	5	13 (65) [a]	5.10 [a]	5.56 (6/108) [a]
28 days									
Q0	26.05 ± 1.02 (24-27)	90 (9/10)	100 (10/10)	0	5	5	15 (75)	7.25	5.20 (8/154)
Q90	26.40 ± 1.32 (24-27)	100 (10/10) [*]	100 (10/10)	0	5	5	15 (75)	8.00	5.33 (9/169)

1 [(No. of male with either 1 or 2 sperm-positive females)/(N = 10)] X100

2 [(No. of males with either 1 or 2 pregnant females)/(N = 10)] x 100. This value also included pregnant females that did not have sperm-positive smears.

3 Total of 20 females were exposed to 10 males, 2 females per 1 male for each mating test

4 [(No. of resorption)/(No. of total fetuses)] X 100; statistical analysis based on % resorptions per litter.

[a] = significant difference ($p < 0.05$) compared with Q0 and Q90

[*] = significant difference ($p < 0.05$) between 14-day and 28-day treatment of Q90 group

4.5 Actions of quercetin on reproductive system in orchidectomized (ODX) rats

4.5.1 Effects of quercetin on relative weight of accessory sex organs in ODX rats

No statistically significant difference of body weight at the first day of study period was noted between 6 groups (Sham, Q0, Q30, Q90, Q270 and Tp groups). The body weight gains were observed in all groups during 14 days of study period.

The relative weight of epididymis plus vas deferens of all orchidectomized rats were significantly lower than the sham rats (Figure 4.19A). In Tp group, although the rats were supplemented with testosterone proprionate (Tp), the epididymis plus vas deferens weight was not fully recovered. Compared with the Q0 group, no significant difference of the relative weight of epididymis plus vas deferens was observed in any quercetin treated groups. Moreover, the relative weight of epididymis plus vas deferens in all quercetin groups was significantly lower than that of the Tp group.

The relative weight of prostate gland of control (Q0) and quercetin treated groups was significantly lower than those of the sham and Tp groups (Figure 4.19B). Supplementation with Tp could completely recover the relative weight of prostate gland and no difference between the sham and Tp groups was found. Compared with the Q0 group, the relative weight of prostate glands showed no significant difference in any quercetin treated groups.

The relative weight of seminal vesicle of control (Q0) and quercetin treated groups was significantly lower than the sham and Tp group (Figure 4.19C). Supplementation with Tp could fully recover the relative weight of seminal vesicle and no difference between the sham and Tp groups was found. Compared with the

Q0 group, the relative weight of seminal vesicles was not significantly different between any quercetin treated groups. The increase of seminal vesicle weight was greater than the increase of prostate gland and epididymis-vas deferens weights, respectively.

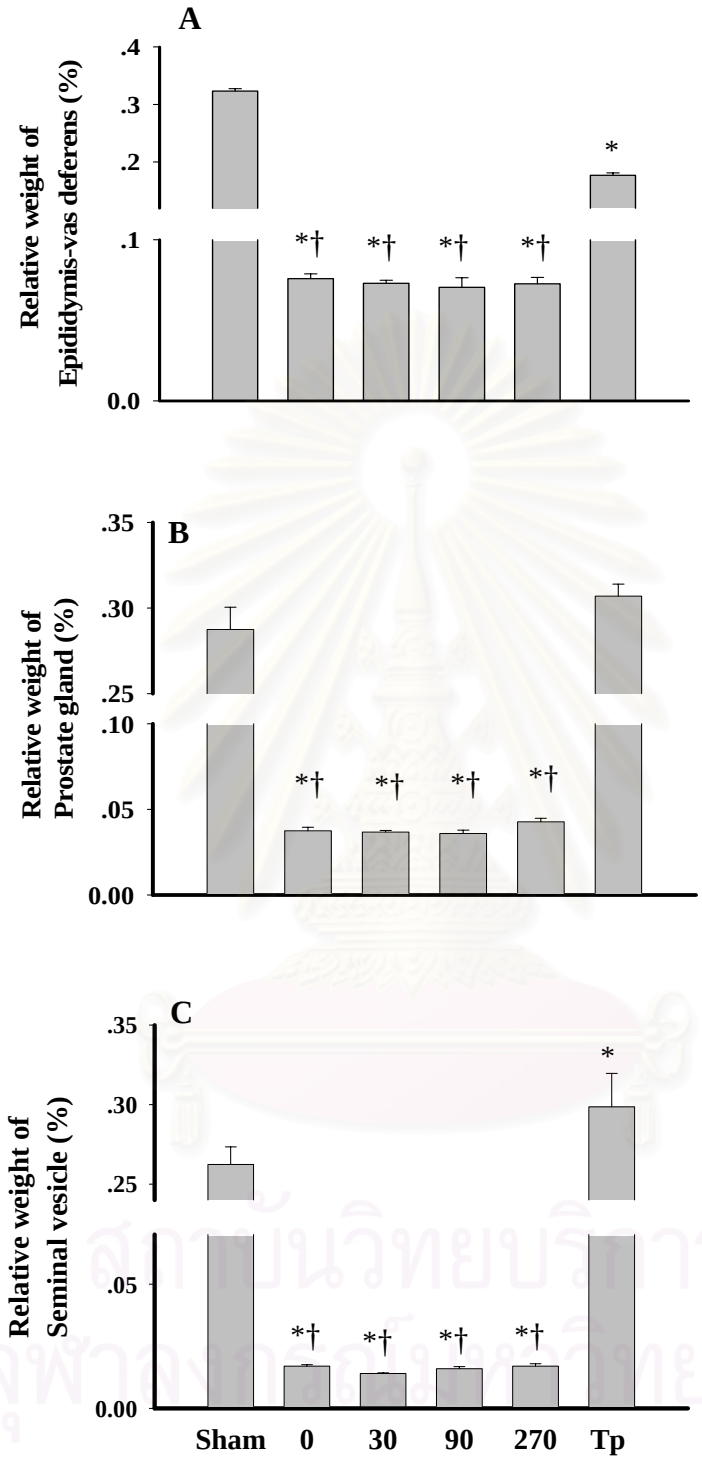

Figure 4.19 Relative weights of male sex organs (%) in orchidectomized rats after subcutaneous injection with quercetin at dose of 0, 30, 90 or 270 mg/kgBW/day or 1 mg/kgBW/day of testosterone proprionate (Tp) for 14 days. Each data point represents the mean ± SEM. * = significant difference ($p < 0.01$) compared with the sham group. † = significant difference ($p < 0.01$) compared with the Tp group.

4.5.2 Effects of quercetin on histology of accessory sex organs in ODX rats

4.5.2.1 Epididymis

The epididymis of the sham group consisted of numerous tubes in which each tube was filled with spermatozoa and fluid (Figure 4.20). In agreement with the slightly increase of epididymis weight, the number of tubes, the presence of spermatozoa and fluid in the epididymis of Tp group were higher than that of quercetin treated groups. However, the presence of spermatozoa and fluid in the tubes were lower than that of the sham group. The epididymal tubes of Tp group were lined by a very tall pseudostratified stereociliated columnar epithelium. Most epithelial cells (or principal cells) had long stereocilia. The numbers of tubes and stereocilia of the control (Q0) and quercetin treated groups were clearly lower than the Tp group. There was no difference in epididymis histology between Q0 and other three quercetin treated groups.

The tubular area, luminal area and tubular thickness of epididymis of the Tp group were significantly higher than the Q0 and quercetin treated groups, but were not different from the sham group (Table 4.8). There was no significant difference in those three parameters between Q0 and quercetin treated groups.

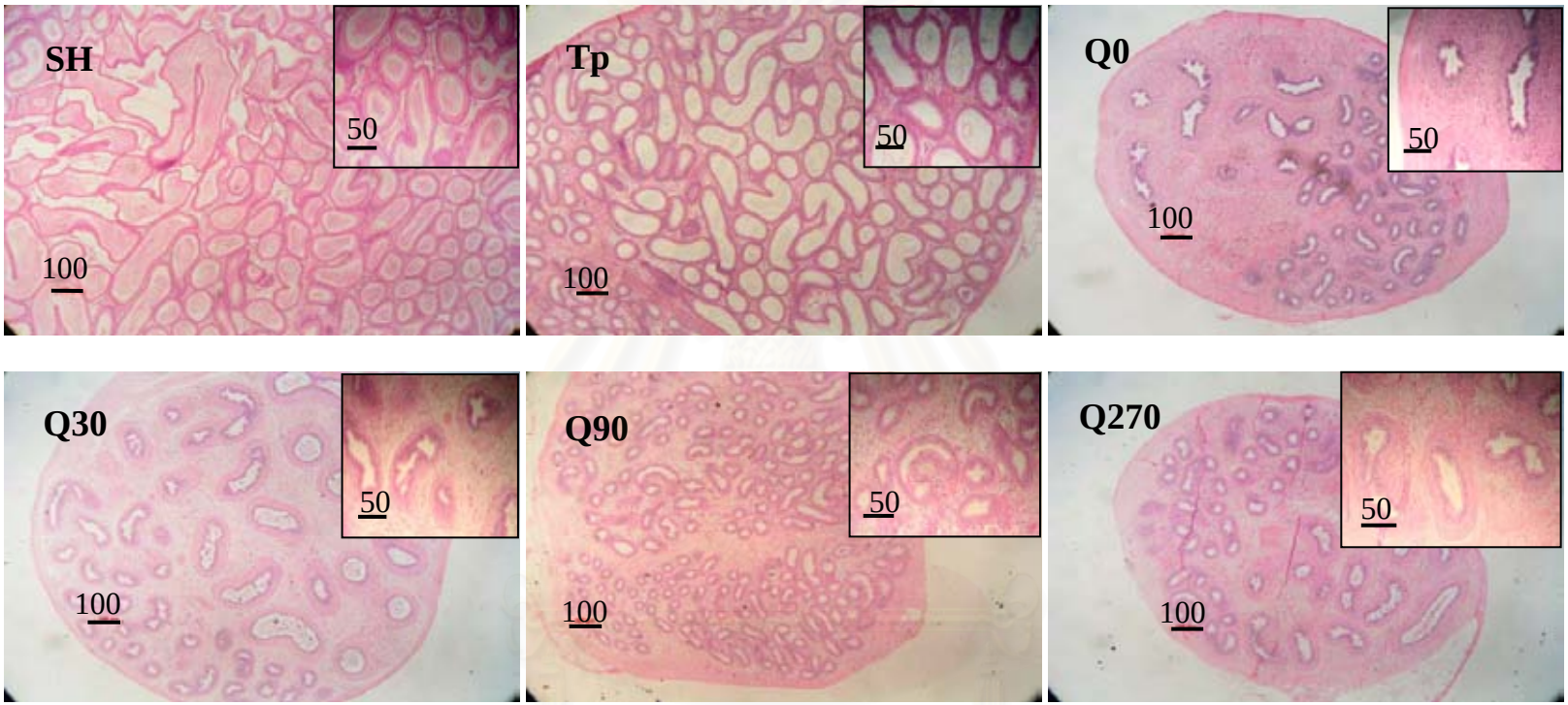

Figure 4.20 Structure of epididymis of orchidectomized rats after subcutaneous injections of quercetin at dose of 0, 30, 90 or 270 mg/kgBW/day (Q0, Q30, Q90 and Q270, respectively) or 1 mg/kgBW/day of testosterone proprionate (Tp) for 14 days (H&E stained, 6 µm section). SH: Sham group. Scale bar = 100 µm.

4.5.2.2 Vas deferens

The mucosa of the vas deferens of the sham group as well as the Tp group formed several longitudinal folds and lined with pseudostratified columnar epithelial cells with long stereocilia (Figure 4.21). In agreement with the higher weights of vas deferens in the Tp and sham groups, the vas deferens thickness of the Tp and sham groups were also higher than those of the quercetin treated groups. In comparison to the Q0 group, the diameter of vas deferens of the Tp group was clearly increased (Table 4.7). There was no difference in vas deferens histology between Q0 and other three quercetin treated groups.

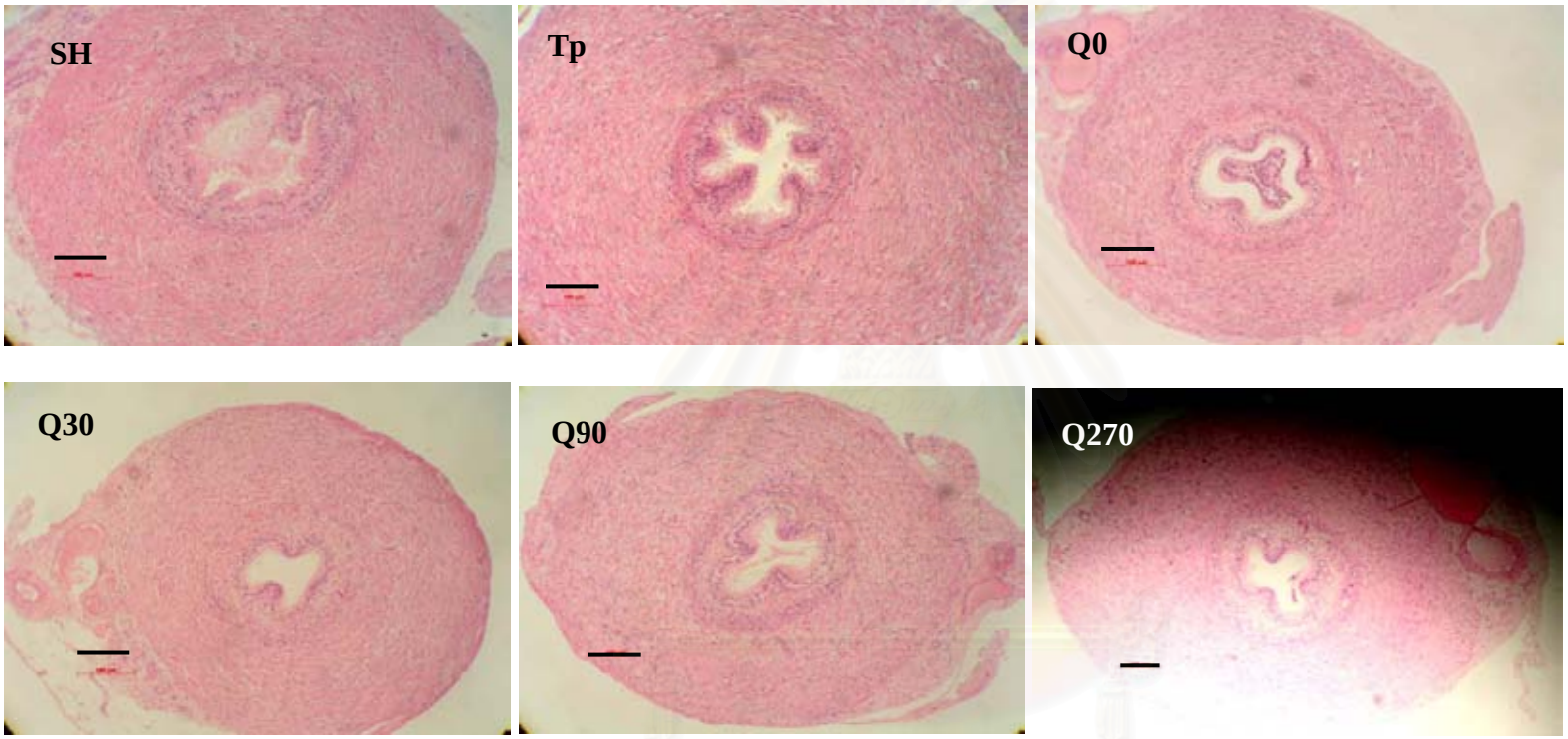

Figure 4.21 Structure of vas deferens of orchidectomized rats after subcutaneous injections of quercetin at dose of 0, 30, 90 or 270 mg/kgBW/day (Q0, Q30, Q90 and Q270, respectively) or 1 mg/kgBW/day of testosterone proprionate (Tp) for 14 days (H&E stained, 6 µm section). SH: Sham group. Scale bar = 100 µm.

Table 4.8 Tubular area, luminal area and tubular thickness of epididymis and diameter of vas deferens in orchidectomized rats after subcutaneous quercetin injection at 0, 30, 90 or 270 mg/kgBW/day for 14 days. Tubular thickness (μm^2) = Tubular area – luminal area.

Groups	Epididymis			Vas deferens
	Tubular area (x 10^3 μm^2)	Luminal area (x 10^3 μm^2)	Tubular thickness (μm^2)	Diameter (μm)
Sham	115.80 ± 12.10	76.62 ± 4.69	32.43 ± 1.54	3.45 ± 1.24
Q0	57.71 ± 3.91 *†	35.67 ± 4.32 *†	25.91 ± 2.09 *†	1.88 ± 1.18 *†
Q30	54.23 ± 7.20 *†	38.43 ± 3.98 *†	24.78 ± 3.43 *†	1.95 ± 1.42 *†
Q90	58.67 ± 6.35 *†	32.56 ± 4.23 *†	22.44 ± 6.01 *†	1.68 ± 2.34 *†
Q270	51.92 ± 4.21 *†	35.89 ± 4.76 *†	26.89 ± 3.63 *†	2.01 ± 1.96 *†
Tp	103.45 ± 7.87	70.86 ± 3.45	31.32 ± 2.34	3.37 ± 1.67

* = significant difference ($p < 0.01$) compared with the sham group

† = significant difference ($p < 0.01$) compared with testosterone propionate group (Tp)

4.5.2.3 Prostate gland

The secretory alveoli of the sham and Tp groups were lined with a layer of tall columnar epithelial cells with a high cytoplasm/nucleus ratio (Figure 4.22). The secretory alveoli in the sham and Tp groups were very irregular shaped. There were high amount of secretory fluids in the lumen of sham group compared with the Tp group. In agreement with the decreased prostate gland weights, the secretory alveoli of the prostate gland in Q0 and quercetin treated groups were found to have irregular shape with no fluid in the lumens. The presence of secretory fluid in lumens of Q0, Q30, Q90, Q270 and Tp were less than those of the sham group. The lumen epithelial cells showed a marked reduction in cytoplasm and their secretory activity became diminished in Q0 and the quercetin treated groups. The number and size of secretory alveoli were clearly increased in Tp group but the presence of secretion was lower than that of the sham group.

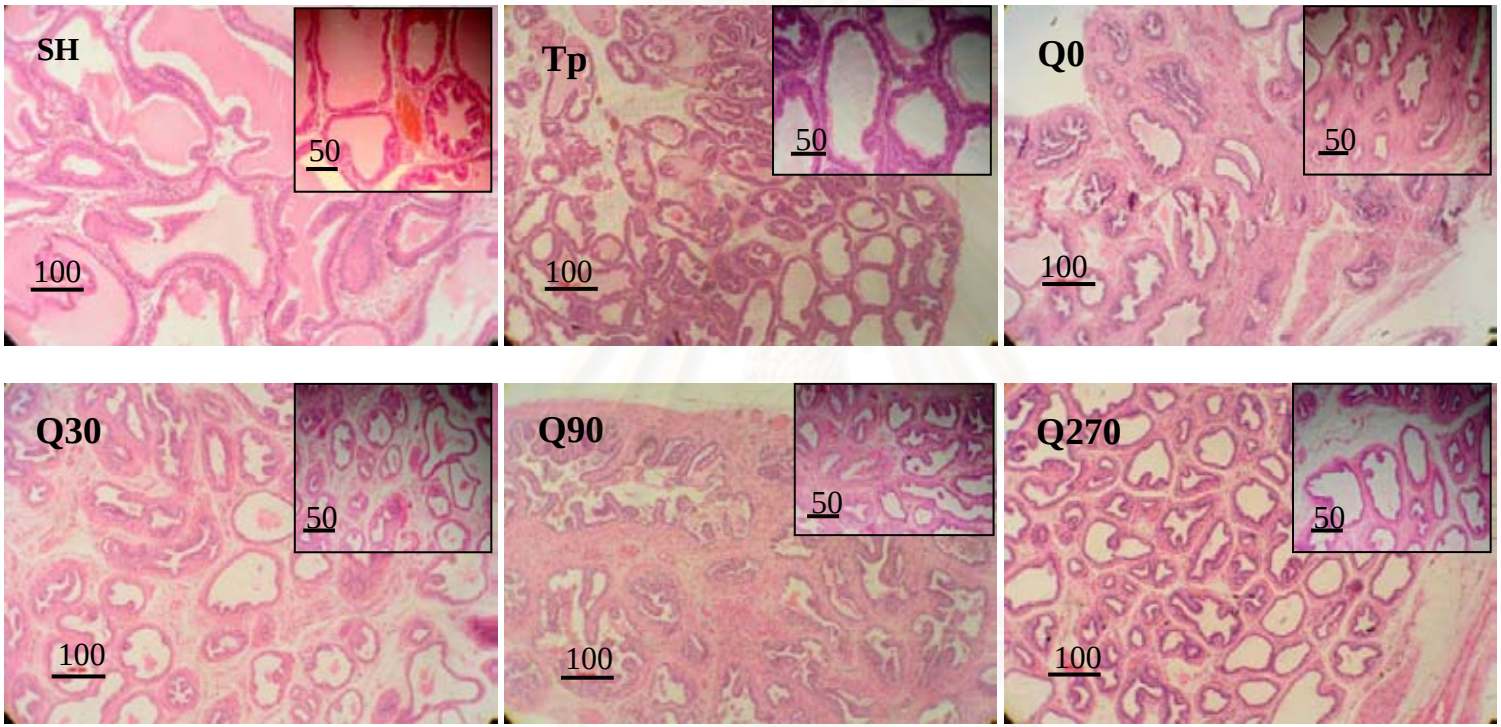

Figure 4.22 Structure of prostate gland of orchidectomized rats after subcutaneous injections of quercetin at dose of 0, 30, 90 or 270 mg/kgBW/day (Q0, Q30, Q90 and Q270, respectively) or 1 mg/kgBW/day of testosterone proprionate (Tp) for 14 days (H&E stained, 6 µm section). SH: Sham group. Scale bar = 100 µm.

4.5.2.4 Seminal vesicle

Seminal vesicle of the sham and Tp groups showed a complex glandular structure and the lumens were highly irregular and recessed giving a honeycomb-like structure (Figure 4.23). The papillary pattern of the epithelial cell lining in these groups consisted of the extensive folding and large amount of seminal fluid. The histology of seminal vesicle of Q0 and quercetin treated groups showed a simple folding pattern with a simple cuboidal epithelial cell lining. There was no difference between the seminal vesicle histology of Q0 and the quercetin treated groups. In agreement with the decrease of seminal vesicle weights, branches of glandular structure in the control (Q0) and quercetin treated groups were clearly less than that of the sham and Tp groups.

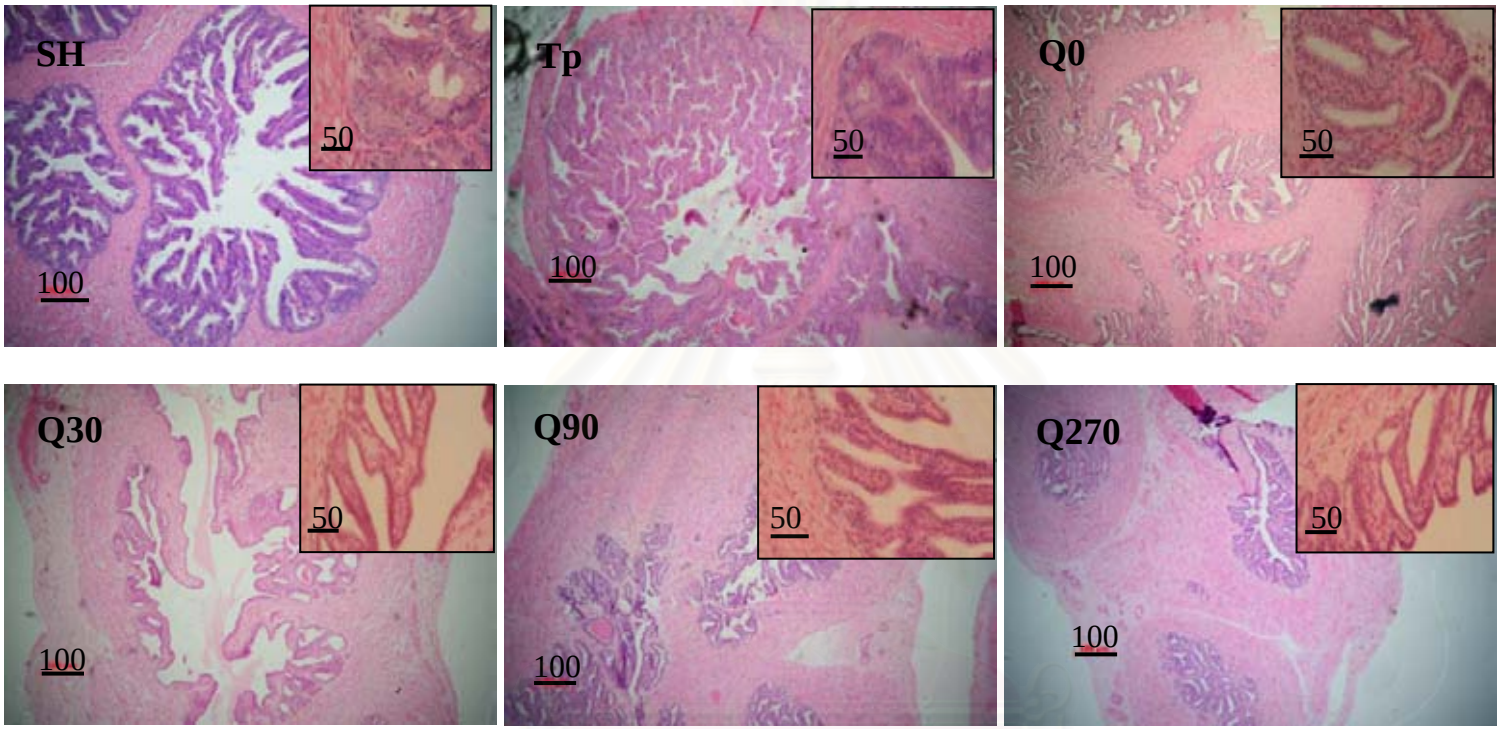

Figure 4.23 Structure of seminal vesicle of orchidectomized rats after subcutaneous injections of quercetin at dose of 0, 30, 90 or 270 mg/kgBW/day (Q0, Q30, Q90 and Q270, respectively) or 1 mg/kgBW/day of testosterone proprionate (Tp) for 14 days (H&E stained, 6 μm section). SH: Sham group. Scale bar = 100 μm.

4.5.3 Effects of quercetin on serum gonadotropin and testosterone levels in orchidectomized rats

Serum LH and FSH levels were found to increase in quercetin treated group, whereas the serum testosterone levels was found to decrease in the sham group (Figure 4.24 (A-C)). The results confirmed that the rats were subjected to a complete orchidectomy. Treatment of quercetin at doses of 30, 90 or 270 mg/kgBW/day to rats did not significantly reduce the increased LH levels, although serum LH levels tended to decrease in Q270 group. Supplementation of Tp could completely suppress the increased LH levels induced by the orchidectomy.

Changes of serum FSH levels were similar to those of LH levels, however, subcutaneous injection of Tp could not completely suppress the increased serum FSH levels as compared with the sham control group. Serum FSH levels of the Q90 and Q270 groups were significantly lower than that of the Q30 group. In agreement with the increased serum LH and FSH levels, serum testosterone levels were found to decrease in all quercetin treated groups. It is important to note that serum testosterone level in Tp group was higher than that of the sham and control group.

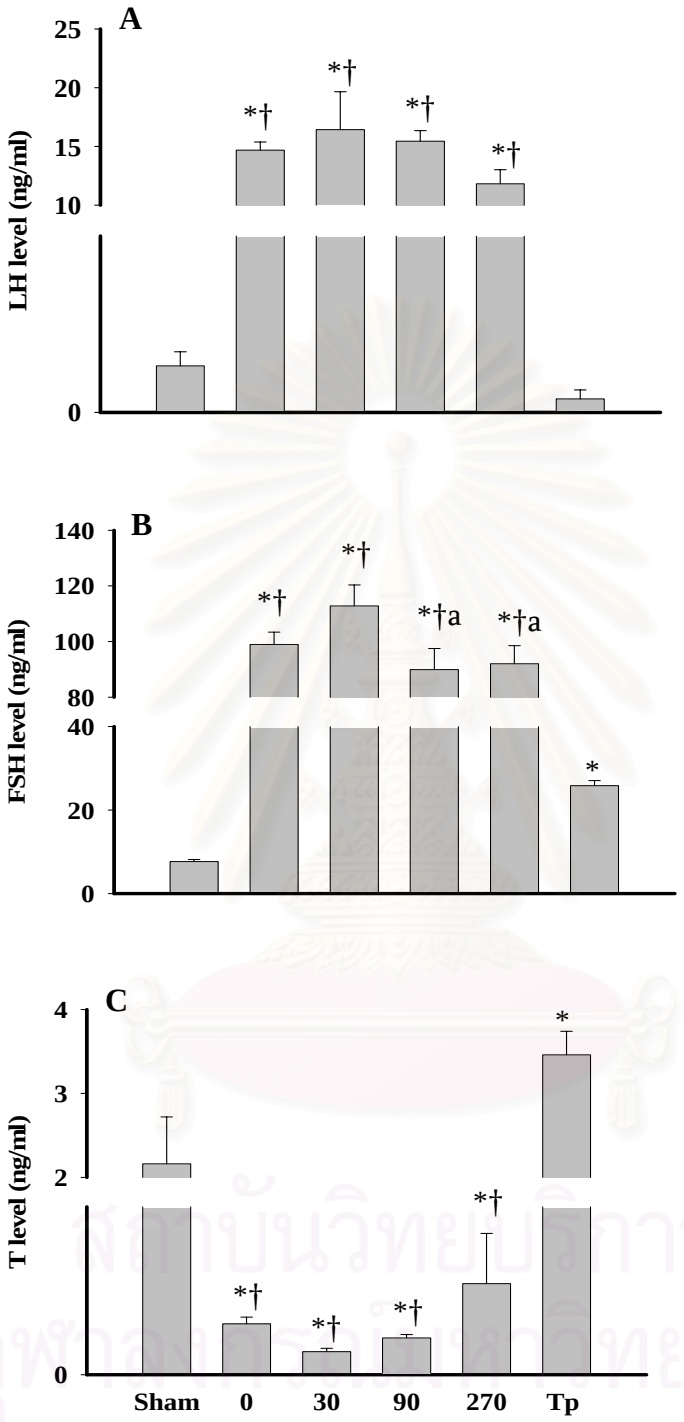

Figure 4.24 The gonadotropin and testosterone levels in orchidectomized rats after subcutaneous injection with quercetin at dose of 0, 30, 90 or 270 mg/kgBW/day or 1 mg/kgBW/day of testosterone proprionate (Tp) for 14 days. Each data point represents the mean ± SEM. * $p < 0.01$ compared with sham group. † $p < 0.01$ compared with Tp group. a = $p < 0.05$ compared with Q30.

CHARPTER V
DISCUSSION AND CONCLUSION

5.1 Effects of age on body weight, spermatogenesis and steroidogenesis

In this study, the weights of body, testis, epididymis-vas deferens, prostate gland and seminal vesicle, and sperm concentration were significantly increased with an advancing age while the histological examinations of these accessory sex organs showed no changes with age. The sperm motility, sperm viability, reproductive hormones (LH, FSH and testosterone levels) and the expression of StAR protein and P450scc were not significantly different among age groups, but they tended to increase with an advancing age.

The age of male rats used in this study was started at 8 weeks old until 12 weeks old. The reported life span of Sprague Dawley rat was 2.5-3 years (Ojeda and Urbanski, 1994). The age at puberty was between 4-7 weeks old and adulthood was started at approximately 7 weeks old or more. Thus, the rats in this study were in the state of young adults or growing stage (Selmanoglu, 2006). The body weight gain of rats during 4 week period of the study (from 8 to 12 weeks old) was approximately 1.2 times which is similar to those of other reports in Sprague-Dawley rats (approximately 1.5 times) (Cooke and Meisami, 1991; Kirby *et al*., 1992), or in Wistar rats (approximately 1.8 times) (Mukerjee and Rajan, 2004).

In male rats, the period of puberty can be estimated by the rapid increase in testicular weight occurring between 4-7 weeks of age. The elongated spermatids, the most mature germ cells, were first seen at 4 weeks old at the proportion of 4% of the testicular cells, while an adult proportion of the elongated spermatids (63% of the testicular cells) was attained at approximately 6 weeks of age and continued raise to 69 % of the testicular cells at 10 weeks old (Bourguignon *et al*., 1990). In agreement

with the present study that the sperm concentration in male rats was increased from 8 weeks to 12 weeks old, previous study showed that, rat, sperm first appear in the seminiferous tubules at about 7 weeks of age (Clermont, 1972; Lee *et al*., 1975).

The production of sperm takes place continuously throughout the reproductive life of the male. In rats, the entire sequence of development from spermatogonia to sperm takes 48-52 days (Leblond and Clermont, 1952). Cycle of spermatogenesis exists with distinct cycle time. Groups of adjacent resting spermatogonia initiate a new cycle about every 12.9 days (Clermont and Harvey, 1965; Rosiepen *et al*., 1994, 1995), constituting one generation of germ cell cohort. During the study period (4 weeks or 28 days), rat's sperm were continuously synthesized (at least 2 cycles) and released to lumen of the seminiferous tubule and transported to the epididymis. By then, the weights of testis and epididymis were increased (1.2 and 1.7 times, respectively) with an advancing age in the present study. The epididymis serves important functions in the transportation, maturation and storage of sperms and enables the sperm to develop motility (Johnson and Everitt, 1985). In agreement with the previous studies, the testis weight gain in male from 8 weeks to 12 weeks old in this study was 1.2 times which is similar to the result of Cooke and Meisami (1991) of 2.3 times in Sprague-Dawley rats and the result of Huhtaniemi *et al*. (1986) of 1.3 times in male Wistar rats. The testis weight of male Sprague-Dawley rats reached a plateau at around 8 to 14 weeks old (Kirby *et al*., 1992). The increase in testicular size at 12-13 weeks of age was associated with increase in numbers of germ cell as well as the total number of Sertoli cells per testis (Hess and Cooke, 1992). The rate of testicular growth was in parallel with the observed increase in body weight (Kirby *et al*., 1992).

Although the weights of testis and epididymis were increased, the histological examination of these sex organs, such as tubular area of seminiferous tubule and tubular area, luminal area and thickness area of epididymis showed no marked differences. It indicated that the increase in weight of these sex organs may cause by the increase in fluid in testes and epididymides. Seminiferous fluid secretion began just after puberty with a sharp increase at approximately 4 weeks old and reaching adult rate at about 6 weeks old (Setchell, 1970; Jegou *et al*., 1982). The seminiferous fluid plays an essential role in the nourishment of germ cells, the transport of tubule secretory products and transport of the newly formed sperm from the tubule to the epididymis (Setchell and Waites, 1975; Purvis and Hansson, 1981). The maturation of sperm required a specific luminal environment, which in turn was created and maintained by testosterone-dependent absorption and secretory activities of the epithelium lining the epididymis (Orgebin-Crist, 1996) that was also dependent on other component of testicular fluid (Sharpe, 1994).

During the study period, the weights of the prostate gland and seminal vesicle were significantly increased with an advancing age which were represented the time course of growth in these organs. It was reported that the prostate gland (Mukerjee and Rajan, 2004) and seminal vesicle (Mukerjee and Rajan, 2006) of Wistar rats progressively increased approximately 4 and 5 times, respectively, from 8 to 12 weeks of age. Major functions of the prostate gland and seminal vesicle include activating the sperm and providing the nutrients that sperm need for motility and viability (Martini and Bartholomew, 1997). Thus, the motility and viability of sperm collected from epididymis in this study were not significant increase with an advancing age. This indicated that the sperm motility and viability require the progressive stimulating sequences through the epididymis and other accessory organs.

The present study showed that basal levels of LH, FSH and testosterone in rats tended to be increased from 8 to 12 weeks old, and agreed with several previous studies (Lee *et al*., 1975; Cooke and Meisami, 1991; Kirby *et al*., 1992). FSH and testosterone are required for initiation of spermatogenesis during sexual maturation (Kerr *et al*., 1992; Spiteri-Grech and Nieschlag, 1993). FSH markedly increases the size of the testes, interacts with the Sertoli cells membrane receptors and causes cAMP production and synthesis of an androgen binding protein (ABP). Furthermore, FSH increases the numbers of LH receptor in Leydig cells (Ketelslegers *et al*., 1978). Sertoli cells provide nutrients and other factors necessary for sperm maturation. The effect of LH on spermatogenesis was thought to be indirect, because the function of LH was to stimulate the synthesis of testosterone. LH stimulates testosterone production by binding to receptors on the plasma membrane of the Leydig cells and activated adenyl cyclase, thus increasing intracellular cAMP. This action enhanced the rate of cholesterol transport by StAR protein and side chain cleavage by P450scc enzyme (Clark *et al*., 1994; Stocco and Clark, 1996a, b; Stocco, 1998). Thus, the expression of StAR protein and P450scc also tended to be increased with an advancing age in this study.

Successful spermatogenesis required androgen production from the Leydig cells (McLachlan *et al*., 2002). Androgens regulate the function of seminiferous tubules, epididymis, prostate gland and seminal vesicle by controlling gene expression via binding to the androgen receptor (Leung *et al*., 2002). Thus, testosterone and DHT levels in rats rise to the maximal levels in adulthood (8-12 weeks old) (Knorr *et al*., 1970; Podesta and Rivarola, 1974).

Testosterone may directly activate androgen receptor or act through local conversion of circulating testosterone to more potent androgen, DHT, by the 5α

reductase enzyme (Russell and Wilson, 1994). DHT has a 10-fold greater affinity for androgen receptor than testosterone (Deslypere *et al*., 1992). To maintain the function of prostate gland and seminal vesicle, it was found that these organs are more responsive to DHT than the circulating testosterone.

5.2 Effects of quercetin on body weight and spermatogenesis

In this study, quercetin in any dosages and any durations of treatment did not affect the body weight gain of male rats compared with that of the control rats (Q0). The increase in body weight was occurred in dependent on an increasing age, not by the quercetin treatment. In agreement with the study of Nakamura *et al*. (2000), the body weight gain of Wistar rats after oral quercetin treatment (10-1000 mg/kgBW) for 21 days was not difference from that of the control rats. However, there was a report showing that the body weight gain was reduced in male rats consumed 40,000 ppm of quercetin per day for 15 weeks (National Toxicology Program, 1992).

In the present study, effects of quercetin on reproductive organs, hormones and sperm quality depended on dose and duration of treatment. The shortest time of quercetin treatment (3 days, subcutaneous injection) at any dosages (30, 90, and 270 mg/kgBW/day) had no effects on reproductive organ weights and histological structure, and sperm quality. However, these effects were observable when the time was prolonged (7, 14 and 28 days) and the dosage was increased (Q90 and Q270).

The testis weights of Q270 group (at day 7 and 14) and epididymis-vas deferens weights of Q90 (at day 28) and Q270 (at day 7 and 14) groups were significantly increased. The histological alterations of these organs were related to the increase in weights. The tubular area of the seminiferous tubules, tubular area, luminal area and thickness area of the epididymis appeared to increase in Q90 (at day

7, 14 and 28) and Q270 groups (at day 7 and 14). Increase in testis weight was probably caused by the fluid and sperm retention in the seminiferous tubules as well as the enlargement of the seminiferous tubule. Increases in sperm retention and weight of testes are associated with the increase in epididymis-vas deferens weights. Increase in sperm retention in the testes is considered to be a consequence of the retention of fluid and sperm in the epididymis which is subsequently caused the dilation of the epididymis tubular lumen and the increased epididymal sperm count and weight. These results are similar to those of Gonzales *et al*. (2001a, 2006) showing that rats treated with Black maca (*Lepidium meyenii*), a potential androgenic plant, for 7 days had an increase in epididymal sperm number and area of the epididymal tubule.

The prostate gland and seminal vesicle weights of Q90 group at day 28 and Q270 group at day 14 were significantly decreased. The histological alterations of these organs were related to the decrease in weights. The prostate lumen was found to be highly dilated and fluid in the lumen and number of tubes were decreased. The seminal vesicle lumen was markedly dilated with reduced branching of the mucosa. It was reported that the lower doses of quercetin (50 and 100 mg/kgBW/day for 10 days) slightly increased the wet prostate weight in rats (Ma *et al*., 2004), which was accompanied by the dramatic dilation of the prostate lumen and the greater retention of fluid. In agreement with the present study, when the dose of quercetin was increased up to 150 mg/kgBW/day (Ma *et al*., 2004), the wet prostate gland weights were decreased. Although the dilation of the prostate lumen still occurred after 150 mg/kgBW/day of quercetin treatment, the fluid retention was slightly reduced (Ma *et al*., 2004). Because the wet weight of organ (the prostate or seminal vesicle) is the combined weight of the organ itself (dry weights) and the fluid (accessory secretion)

inside, the results from this study suggested that the effect of test substances on sex organs should not be solely evaluated by weight changes but should also be evaluated by histological changes. In addition to the luminal dilation, the prostatic epithelial height, a known androgen dependent characteristic (Gonzales *et al*., 2005), appeared to be decreased by the Q90 or Q270 treatment for 28 and 14 days, respectively.

The response in sperm motility, sperm viability and sperm concentration to quercetin treatment was faster and at a greater degree than responses in weights and histological structure of accessory sex organs. Sperm motility of Q90 (at day 7, 14 and 28) and Q270 groups (at day 7 and 14), sperm viability of Q90 and Q270 groups (at day 7 and 14) and sperm concentration of Q30 (at day 7 and 14), Q90 (at day 7, 14 and 28) and Q270 groups (at day 7 and 14) were significantly increased. Ma *et al*. (2004) reported that the levels of serum quercetin metabolites reached a plateau on the 6^{th} day of daily quercetin feeding in male rats. In the present study, the increase in sperm quality (motility, viability and concentration) could be detected within 7 days of treatment at higher doses (Q90 and Q270) of quercetin. Gozales *et al*. (2006) reported that the epididymal sperm count in rats was increased after only 1 day of Black maca (*Lepidium meyenii*) feeding and the subsequent sperm count in the vas deferens was increased after 3 days of feeding. They described that the increase in sperm count after Black maca treatment was caused by changes in regulatory mechanism of the distribution of sperm produced in testis rather than the higher production of sperm. Since the entire sequence of development from spermatogonia to sperm takes 48-52 days in rats (Leblond and Clermont, 1952) and a cohort of resting spermatogonia initiates a new cycle every 12.9 days (Clermont, and Harvey, 1965; Rosiepen *et al*., 1994, 1995), the increase in sperm concentration in epididymis after quercetin treatment (Q30, Q90 and Q270) only 7-28 days should be caused by

the distribution of sperm from testis to epididymis rather than the increase sperm production.

The improved sperm motility after quercetin injection in this study was agree with a result of Hammerstedt *et al*. (1988) that quercetin administration enhanced an ejaculated bovine sperm motility. However, this *in vivo* study disagrees with the results of previous reports obtained from the *in vitro* study (Trejo *et al*., 1995; Khanduja *et al*., 2001). In the previous *in vitro* study, the human semen incubated with quercetin showed decrease in sperm motility (at 5-200 μM) and sperm viability (50-100 μM) and decrease of Ca^{2+}-ATPase activity, a key enzyme involved in the regulation of sperm motility (Khanduja *et al*., 2001). The viability of spermatids incubated with quercetin was also decreased (Trejo *et al*., 1995). One possible explanation for this disagreement is that in the *in vivo* study the quercetin may act through other sex organs (i.e., stimulating the testis or epididymis in the present study), with no directly action on sperm inside the testis or epididymis. It was previously reported that the quercetin may act dose-dependently as either an agonist of endogenous steroids at low doses or an antagonist at high doses (Maggiolini *et al*., 2001).

It is possible that the increase in epididymal sperm quality in this study may be due to the protective effect of quercetin on sperm from the oxidative injury by encouraging scavengers of reactive oxygen species or an antioxidant activity of quercetin in the epididymis (Zini and Schlegel, 1997). Reactive oxygen species (ROS) are produced in normal cellular metabolism (Aitken, 1994) and also found in human sperm (de Lamirande and Gagon, 1992; Aitken, 1994). ROS are reportedly associated with sperm damage and reduction in sperm motility (Plante *et al*., 1994; Perinaud *et al*., 1997) via depletion of intracellular adenosine triphosphate and the

subsequent decrease in the phosphorylation of axonemal proteins (Gagnon *et al*., 1991; Agarwal, 2005). The previously reported example of ROS affected sperm function includes hydrogen peroxide. Hydrogen peroxide caused a decrease in the antioxidant defenses of the sperm which ultimately lead to the peroxidation of the membrane phospholipids by ROS (Griveau *et al*., 1995). It changed the membrane fluidity and integrity by inducing an accumulation of lipid peroxide and disrupted sperm motility and viability. Quercetin has 3 hydroxyl groups which make the compound a potential free radical scavenger and antioxidant (Jovanovic *et al*., 1994; Terao *et al*., 1994; Hu *et al*., 1995). Therefore, quercetin may increase sperm motility and sperm viability via acting as antioxidant in epididymal sperm.

Since the sperm quality, weights of testis and epididymis-vas deferens were increased and their histological structures were changed by quercetin treatment in this study, therefore, quercetin seemed to acquire an androgenic activity.

5.3 Effects of quercetin on steroidogenesis

Serum LH levels of Q90 (at day 7, 14 and 28) and Q270 groups (at day 3, 7 and 14), FSH levels of Q90 (at day 7 and 28) and Q270 groups (at day7 and 14) and serum testosterone levels of Q270 (at day 3 and 7) were significantly decreased after quercetin treatment in the intact male rats. The decrease in serum testosterone levels after Q270 injection disagreed with the previous study which reports that quercetin increased serum testosterone levels in rats (Ma *et al*., 2004). In the present study, quercetin suppressed the secretion of LH and FSH after Q90 and Q270 injection, and subsequently caused a reduction of testosterone production. Therefore, quercetin seems to have an androgenic activity on the HPG axis. The study of Gonzales *et al*.

(2001b) found that the Black maca (*Lepidium meyenii*), another potential androgenic plant, tended to decrease serum LH, FSH and testosterone levels in men.

Leydig cell steroidogenesis is regulated by LH and involves the increase in the formation of the second messenger cAMP signal transduction pathway via the LH receptor. LH increases the gene transcription of steroidogenic acute regulatory (StAR) protein and several steroidogenic enzymes (de Kretser and Kerr, 1994; Payne *et al*., 1996). The expressions of StAR protein and P450scc were not changed after quercetin treatment, except for Q90 at day 28 which showed a decreased expression of P450scc. Thus, the reduction in testosterone levels was seemingly not related to the reduction of testosterone synthesis via LH stimulation.

The reduction of testosterone levels of Q270 group was related to the reduction of the weight of prostate gland and seminal vesicle. The seminal vesicle was the most sensitive organ to testosterone stimulation (Stroheker *et al*., 2003). Low seminal vesicle weight is correlated with low serum testosterone levels (Chung *et al*., 2005). Rat prostate gland contains androgen receptors which are the direct target of androgen action (Prins *et al*., 1991) and mainly dependent on testicular androgens (Reiter *et al*., 1995). In the prostate gland, seminal vesicle and male accessory organs, testosterone is converted to DHT before exerts its action (Higgins and Burchell, 1978; Lieber et al., 1980). DHT has a 10-fold greater affinity for androgen receptor than testosterone (Deslypere *et al*., 1992). Prostate gland and seminal vesicle are more responsive to DHT than circulating testosterone. Ma and co-worker (2004) found that quercetin treatment resulted in a biphasic change in serum DHT concentration, which was slightly increased at low dose (50 mg/kgBW), but decreased at high dose (150 mg/kgBW) (Ma *et al*., 2004). Thus, quercetin at high dose (Q270)

may inhibit 5α reductase activity and decrease DHT levels, resulted in a reduction in weights of the prostate gland and seminal vesicle.

5.4 Effects of quercetin on fertility

The percentage of male with sperm positive female and male fertility index in Q270 group was significantly lower than the other 2 groups (Q0 and Q90). Thus, the number of pregnant females was lower in Q270 group. The number of fetus per litter was also significantly decreased in Q270 group whereas the percentage of resorptions was significantly increased. Changes of these parameters in Q90 group were not found when compared with the control group (Q0) at both of 14- and 28-day treatments. However, the percentage of male with sperm positive female in Q90 group at day 28 was significantly higher than that of Q90 group at day 14. The results agree with Aravindashan *et al*. (1985) reported that a higher dose of quercetin treatment (300 mg/kgBW, two injections) reduced the fertility rate of male rats during the first two matings with female rats, but the fertility was recovered to be comparable to the control group afterward.

It is important to note that the sperm maturation requires many progressive stimulating sequences. It is not only passed through the epididymis but also stimulated by other accessory sex organs, e.g. prostate gland and seminal vesicle. Although the epididymal sperm quality was found to be increased after Q270 treatment, the male fertility was still decreased. The reduction of male fertility after quercetin treatment was possibly related to the reduction of the weights of prostate gland, seminal vesicle and testosterone levels observed in Q270 treatment of the present study. In rats, any increases in serum testosterone levels or treatment with androgens are associated with an increased secretory activity of the seminal vesicle

(Higgins and Burchell, 1978; Zanato *et al*., 1994; Fawell and Higgins, 1984) and an increased seminal vesicle weight (Almemnara *et al*., 2000). The seminal vesicle secretes a great variety of products (Almemnara *et al*., 2000). The seminal vesicle secretion is the important factor allowing all sperm to be in contact with the ingredients in the semen which may promote sperm motility, increase stability of sperm chromatin and suppress the immune activity in the female reproductive tract to avoid rejection of sperm and embryo (Gonzales *et al*., 2001a). The prostate gland secretes a thin, milky fluid that contains citrate ion, calcium, phosphate ion, a clotting enzyme, and profibrinogenolysis. The prostatic fluid is important for fertility (Burkitt *et al*., 1993; Jones, 1997). The constituents of prostatic secretion provide sperm with an optimal environment following ejaculation into the hostile milieu of the vagina (Burden *et al*., 2006). The function of these accessory sex organs was important for the successful fertilization of sperm with the ovum (Burkitt *et al*., 1993; Jones, 1997). Thus, the reduction of weights and alterations of histology of prostate gland and seminal vesicle by quercetin treatment may lead to the reduction of fertility in the intact rats. Although the reduction of weights of the prostate gland and seminal vesicle was also observed in the lower dose of quercetin treatment (Q90 for 28 days), the fertility in male rats was still increased when the sperm-positive female is used as an indicator. It is possible that those reductions after Q90 treatment were not severe and might be in the lower level of normal range.

5.5 Actions of quercetin on reproductive system in orchidectomized rats

After orchidectomy for 14 days, the rats showed increased FSH and LH levels, decreased testosterone levels and decreased weights of accessory sex organs including epididymis-vas deferens, prostate gland and seminal vesicle in comparison with those

of the sham rats. These results confirmed that the rats were subjected to the complete removal of the testes as reported in previous studies (Ramirez and McCann, 1965; Gay and Bogdanove, 1969; Swerdloff and Walsh, 1973). However, these changes were not recovered after quercetin treatment. The results indicate that quercetin may have very low or no androgenic effects on accessory sex organs and gonadotropin secretion in ODX rats.

After testosterone propionate treatment, a positive control for this study, serum LH and FSH levels were decreased, serum testosterone levels were increased and weights of the epididymis and seminal vesicle were also recovered. The results indicate that Tp can reverse these effects on accessory reproductive organs and gonadotropins levels caused by ODX. The serum testosterone levels determined in Tp group was very high. The determined testosterone level was possibly the Tp that was injected to the rats and able to cross-react with the antibody of testosterone assay by RIA used in this study. However, the inhibitory effect of Tp on FSH levels was not complete, probably because the inhibition of FSH levels at the pituitary was caused by both testosterone and inhibin (Hadley, 2000).

The increase of seminal vesicle weight in Tp group was greater than the increase of prostate gland and epididymis-vas deferens weights, respectively. It indicates that the response of seminal vesicle to testosterone was higher than prostate gland and epididymis and supports the statement that the seminal vesicle is the most sensitive organ for testosterone stimulation (Stroheker *et al*., 2003).

Since effects of quercetin on weights and histological structures of accessory sex organs and on HPG axis were not detectable in the orchidectomized rats, it clearly showed that quercetin has no androgenic activity by itself, and it may potentiate the activity of endogenous androgens or exhibits its mild androgenic activity via other

pathways such as the antioxidant activity or estrogenic activity or anti-estrogenic activity.

It is well known that estrogens were also important for male reproduction. Estradiol could reduced sperm motility, serum LH, FSH and testosterone levels, weights of testis and accessory sex organs in rats (Gill-Sharma *et al*., 2001). Quercetin activates the ERβ and ERα for 4.5 and 1.7 folds, respectively, higher than that of estradiol (Van der Woude *et al*., 2005). It was reported that quercetin stimulated proliferation of ER-positive cells, MCF-7 and T47D cells, suggesting this effect to be ER-dependent (Van der Woude *et al*., 2005). Quercetin will exhibit estrogenic or anti-estrogenic activity depended on dosage. Quercetin at the dose of 2 μM stimulated the proliferation of human adipose tissue-derived stromal cells (hADSC) or estrogenic effects, but at dose of 5 and 10 μM of quercetin inhibited the proliferation of hADSC or anti-estrogenic effects (Kim *et al*., 2006).

In vitro study, quercetin could decrease hCG-stimulated testosterone secretion by inhibiting the activity of 3β-HSD and 17β-HSD in isolated Leydig cells (unpublished data). These results were similar to the result of Whitehead and Lacey (2003) found that quercetin could reduce the progesterone secretion by inhibiting the 3β-HSD activity in H295R cells and inhibiting 17β-HSD activity in granulose-luteal cells (Lacey *et al*., 2005). Therefore, the reduction in testosterone levels in the present study may be also related to the inhibiting activity of steroidogenic enzymes.

From these results, indicate that the quercetin may possess both a central effect for suppressing gonadotropin secretion and a local effect for stimulating spermatogenesis and steroidogenesis in Leydig cells (*in vitro* study, unpublished data). The central effect on the hypothalamus-pituitary axis may cause by the

quercetin itself, because quercetin can pass through the blood brain barrier (Youdim *et al*., 2004).

Based on these results, use of quercetin (Q90) for long duration (day 28) should be one of alternative choices for treatment of male fertility because it can increase sperm quality and fertility without undesirable side-effect on sex steroid hormone synthesis. In addition, it has been reported that quercetin could inhibit the specific binding between dioxins (toxic members of halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons) and aryl hydrocarbon receptor (AhR) (Poland *et al*., 1976; Poland and Knutson, 1982; Fukada *et al*., 2007). Binding of dioxins, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin), to AhR can cause defects in reproductive system of male rats (Gibbons, 1993; Baba *et al*., 2005). It leads to a decrease of sperm count in the caudal epididymis, weights of epididymis prostate gland and seminal vesicles (Gray *et al*., 1995; Faqi *et al*., 1998). Therefore, the supplement of quercetin, which can be found in many plants and plants products, such as onions, apples, tea, cherries, berries, grapes and plums should be a by-side effect to improve fertility in men who live in the environmental toxin.

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APPENDICES

APPENDIX I

General Chemicals

- Absosulte Ethanol (E. Merck, Damstadt, Germany)
- Agarose gel (Research Organics, USA)
- Boric acid (Research Organics, USA)
- Diethyl ether (E. Merck, Damstadt, Germany)
- Disodium hydrogen phosphate anhydrous (Na_2HPO_4) (BDH Chemical Ltd., England)
- DNA Ladder Marker 100 bp (SibEnzyme, Russia)
- Eosin (E. Merck, Damstadt, Germany)
- Ethanol 95% (E. Merck, Damstadt, Germany)
- Ethidium bromide (Bio Basic, Inc., USA)
- Ethylene diamine tetra-acetic acid (EDTA), (Serve Feinbiochemica GmbH & Co., USA)
- Formalin or 40% formaldehyde (E. Merck, Damstadt, Germany)
- Glacial acetic acid (E. Merck, Damstadt, Germany)
- Glycerine (E. Merck, Damstadt, Germany)
- Glycerol (BDH Laboratory Supplies, England)
- Hematoxylin (E. Merck, Damstadt, Germany)
- Maxime RT-PCR PreMix Kit (catalog# 25131, iNtRON Biotechnology, Korea).
- n-butyl alcohol (absolute) (E. Merck, Damstadt, Germany)
- PCR Purification Kit (catalog# 28104, Qiagen, Germany)
- Picric acid (E. Merck, Damstadt, Germany)
- Primers or olionucleotides (Bioservice Unit, Thailand)
- Quercetin (catalog# 33,795-1, Aldrich, Germany)
- RNA Extraction Kit (e-Zi RNA extraction kit, SunolinCorporation, Thailand).
- RNAlater®: RNA stabilization solution (catalog# 7024, Ambion, Japan)
- Testostertone proprionate (Sigma, Germany)
- Tris-(Hydroxymetyl)-aminomethane, (Pharmacia Biotech, USA)

Chemicals for radioimmunoassay

- FSH standard : National Institute of Diabetes and Digestive and Kidney Disease (NIDDK), NIH, Torrance, CA, USA

(Batch number NIDDK-rFSH-RP-2 (AFP-4621B))

- antiserum to FSH : NIDDK, USA

(Batch number NIDDK-anti-rFSH-S-11 (AFPO-972881))

- FSH tracer : NIDDK, USA

(Batch number NIDDK-rFSH-I-7 (AFP-11454B))

- LH standard : NIDDK, USA

(Batch number NIDDK-rLH-RP-3 (AFP-7187B))

- antiserum to LH : NIDDK, USA

(Batch number NIDDK-anti-rLH-S-11 (AFPO-972881))

- LH tracer : NIDDK, USA

(Batch number NIDDK-rLH-I-7 (AFP-11536B))

- Testosterone standard : WHO Matched Reagent Programme, Switzerland.

(Batch number K079810)

- antiserum to Testosterone : WHO Matched Reagent Programme, Switzerland.

(Batch number K888510)

- Testosterone tracer (1,2,6,7-3H) : Amersham International, PLC, England.

(Lot TRK 402)

Equipments

- Autoclave model: Conbraco (Conbraco Ind. Inc., USA)
- Automatic micropipette: P10, P20, P100, P200 and P1,000 (Gilson, France)
- Cuvette: model 0.7-0.8 ml (Starna, England)
- Digital camera (Canon, USA)
- Dynac centrifuge (Clay Adams, Becton Dickinson and Company, USA)

- Electric balance: model Denver instrument APX-203 (Denver instrument, USA)
- Electric UV transilluminator (Ultra Lum Inc., USA)
- Electrophoresis chamber set: model Mupid (Advance Co. Ltd., Japan)
- Freezer -20 °C (Sanyo, Thailand)
- Hemocytometer : model Improved Neubauer (Boeco, Germany)
- Light microscopy (Olympus, Japan)
- Light microscopy (Carl Zeiss, Germany).
- Microcentrifuge tubes (0.5 and 1.5 ml) (Sarstedt, Germany)
- Microcentrifuge: model Centrifuge pico (Sorvall, Kendro laboratory products, Germany)
- Microcentrifuge: model Coolspin 2 centrifuge, MSE (International Equipment Company, USA)
- Microscope glass slide (catalog# 7105, Sail Brand, China)
- Microtome: model 820 serial 66305 (American Optical, Scientific Instrument Division, Buffalo, New York, USA)
- Microtome blade (S35, USA)
- Microwave oven: model Sharp carousel R7456 (Sharp, Thailand)
- Paraffin dispenser (Ashcroft, USA)
- PCR HEPA Safety cabinet, (Augusta, Lio Lab Ltd. Part, Thailand)
- PCR machine: model Gene Amp PCR system 9700, (Applied Biosystem, Singapore)
- PCR tube (Thin-wall microcentrifuge tube (0.2 ml)
- pH meter, model 215 (Denver instrument, USA)
- Pipette tips (0.1-10 µl) (Sorenson Bioscience, Inc, USA)
- Pipette tips (200 and 1,000 µl) (Bioline, USA)
- Power supply: model EC 570-90 LVD CE (E-C Apparatus corporation, USA)
- Spectrophotometer: model: Ultraspec II (LKB biochem, England)
- Spectrophotometer: model DU600 (Beckman Coulter™, USA)
- Syringe 1 and 5 ml and needle #18, 23, 21 (Nipro, Corp. Ltd., Thailand)
- Ultrasonic cleanser (Right A weight, WM, Denver, USA)
- Vortex Mixer: model KMC-1300V (Vision Scientifig Co. Ltd., Korea)
- Whatman laboratory sealing film (Whatman International Ltd. England)

APPENDIX II

Tissue Preparation for Light Microscope

1. Fixation and paraffin technique

Fixative solution (Bouin's solution)

1. 1.2 % Picric acid	75 ml
2. 40 % Formalin	25 ml
3. Glacial acetic acid	5 ml

The testes, epididymis, vas deferens, prostate gland and seminal vesicle were dissected and fixed in Bouin's fixative solution for at least 18 hours. Then they were washed with 70 % ethanol in order to remove picric acid. After being washed, the tissues were dehydrated with serial concentration of ethanol (70%, 90% and 95%) and n butanol, respectively. Each process was performed for 6 hour except at 95% ethanol, which was repeated twice for 6 hour and at n-butanol which was performed for 1 hour. Then the tissues were cleared by xylene for 1 time and infiltrated with liquid paraffin for 3 times (1 hour per each process). Finally, the tissues were embedded in paraffin block.

2. Sectioning

Paraffin blocks were cross section cut at 6 μM thick with microtome.

3. Staining

Tissues sections were stained with Mayer's Hematoxylin and Eosin.

Staining solution

Mayer's Hematoxylin solution

Hematoxylin crystals	1 g
Sodium iodate ($NaIO_3$)	0.2 g

Ammonium or potassium alum	50 g
Citric acid	1 g
Chloral hydrate	50 g
Distilled water	1000 ml

Preparation The Hematoxylin, alum and sodium iodate were dissolved in distilled water and stand the mixture was left overnight at room temperature. The chloral hydrate and citric acid were added, mixed and boiled for 5 minutes. The solution was cooled down and filtered.

1% stock alcoholic Eosin

Eosin Y	1 g
80% alcohol	80 ml
Glacial acetic acid	0.5 ml
Distilled water	20 ml

Preparation Eosin Y was dissolved in 80% alcohol, glacial acetic acid and distilled water.

Staining procedure of Hematoxylin and eosin

Paraffin sections were transferred with hair brush to a glass slide with drops of egg albumin solution and warmed on a heater at 40-50°C. The slides were placed in xylene solution for 2 times (3-5 minutes each). The sections were hydrated by passing through n-butanol, 95%, 90% and 70% ethanol respectively for 1-2 minutes each. Tissues were immersed into tap water in order to wash out the alcohol and rehydrated the tissues. Tissues were stained with hematoxylin for 5 minutes and immersed into tap water in order to wash out excess hematoxylin. The tissues were differentiated in order to remove excess color of hematoxylin and increase degree of

difference among stained organelles using 0.5% acid alcohol for 5-7 second and then washed with tap water for 2-3 times. The tissues were immersed in saturated lithium carbonate in water for 3-5 second and then washed off with tap water. The sections should be examined under microscope. The nuclei of the cells with a properly stained should be deep blue color while the background should be shown only weak color. The sections were washed quickly in ethanol, differentiated and dehydrated in a serial concentration of ethanol (70%, 95% and 100% ethanol respectively). The sections were immersed in 95% ethanol and stained with eosin solution for 10 second. The tissues were cleared with xylene for 2 times (5 minutes each). The sections were mounted on slide with permount solution and covered with the coverslip.

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APPENDIX III

Determination of Serum Hormone Levels by Radioimmunoassay Technique

A. Gonadotropin Determination

1. Reagent preparation

1.1 0.5 M PBS-0.1% NaN_3 pH 7.6

$Na_2HPO_4.12H_2O$ (14.5071 grams), $NaH_2PO_4.2H_2O$ (1.4825 grams), NaCl (8.72 grams), and NaN_3 (1 grams) were dissolved in 1 liters of di-distilled water, and then the pH was adjusted to 7.6. The solution was stored at 4 °C where it should be stable for 1 month.

1.2 0.5 M PBS-0.1% NaN_3 -0.05 M EDTA-1% NRS pH 7.6

The assay buffer of 0.5 M PBS-0.1% NaN_3 was added with EDTA (18.612 g/L), then the pH of this mixed solution adjusted to 7.6 by a dropwise addition of 5 M NaOH. Then Normal rabbit serum (NRS) was added and mixed.

2. Preparation of FSH tracer, antiserum and standards

2.1 FSH tracer

Lyophilized form of FSH tracer (Batch number NIDDK-anti-rFSH-I-7 (AFP 11454B)), 100 μg/ampule, was dissolved in 1 ml of assay buffer, and then the solution was aliquoted into vials (20-25 μg/vial). The solution was stored at 4 ºC where it should be stable for 4-6 months. FSH tracer was added with 0.05 M PBS-0.1% NaN_3-0.1% BSA until the count per minute (CPM) value was 4,000-5,000 cpm/50 μl.

2.2 FSH antiserum

FSH antiserum (NIDDK-anti-rFSH-S-11 (AFPO 972881)) was dissolved in 1 ml of 1:12.5 of 2% normal rabbit serum (NRS): assay buffer, then the solution was lyophilized. The lyophilized form of FSH antiserum was mixed with 1 ml of di-distilled water and then used as a stock solution. This solution was stored at 0 °C. When working solution was required, stock solution of FSH antiserum was added with 0.05 M PBS-0.1% NaN_3-0.05 M EDTA-1% NRS (pH 7.6) and mixed until the final concentration was 1:350,000.

2.3 FSH standard

Lyophilized form of FSH standard (NIDDK-rFSH-RP-2 (AFP-4621B)) was dissolved in 1 ml of 1% BSA phosphosaline buffer to yield the concentration of 1 μg/ml. Then 100 μl of the solution was aliquoted into each vial and stored at 0°C where it should be stable for 3-5 months. The concentrations of FSH standard in serial dilution were 0.019, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5 and 10 ng/100 μl /tube.

3. Preparation of LH tracer, antiserum and standards

3.1 LH tracer

Lyophilized form of LH tracer (NIDDK-rLH-I-5 (AFP-11536B)), 100 μg/ampule, was dissolved in 1 ml of assay buffer, and then the solution was aliquoted into vials (20-25 μg/vial). The solution was stored at 0 °C where it should be stable for 2-3 months. LH tracer was added with 0.05 M PBS-0.1% NaN_3-0.1% BSA until the cpm value was 4,000-5,000 cpm/50 μl.

3.2 LH antiserum

LH antiserum (NIDDK-anti-rLH-S-11 (AFPO 972881)) was dissolved in 1 ml of 1:18.75 of 2% normal rabbit serum (NRS): assay buffer, then the solution was lyophilized. The lyophilized form of LH antiserum was mixed with 1 ml of di-distilled water and served as a stock solution. This solution was stored at 0 °C. When working solution was required, stock solution of LH antiserum was added with 0.05 M PBS-0.1% NaN_3-0.05 M EDTA-1% NRS (pH 7.6) and mixed until the final concentration was 1:130,000.

3.3 LH standard

Lyophilized form of LH standard (NIDDK-rLH-RP-3 (AFP-7187B)) was dissolved in 1 ml of 1% BSA phosphosaline buffer to yield the concentration of 100 ng/ml. Then 100 μl of solution was aliquoted into each vial and stored at 0° where it should be stable for 3-5 months. The concentrations of LH standard in serial dilution were 1.9, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1,000 pg/100 μl /tube.

4. Radioimmunoassay procedures for gonadotropins (FSH, LH)

Pipette 100 μl of standard (in 0.05 M phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA)) or 100 μl of sample into 10 x 75 mm disposable glass tube. Then, add 50 μl of anti-rat FSH (NIDDK anti-rat FSH-S-11, 1:350,000) or anti-rat LH (NIDDK anti-rat LH-S-10, 1:130,000) (in 0.05 M PBS containing 0.4% normal rabbit serum and 0.05 M EDTA. Incubate at 4 °C for 24 h for FSH, and 48 h for LH.

Add 50 μl of ^{125}I-rat FSH (NIDDK rat FSH-I-7) or ^{125}I-rat LH (NIDDK rat LH-I-7) (in 0.05M PBS containing 1% BSA) and incubate at 4 °C for 96 h for FSH or 48 h for LH. Then add 50 μl (for LH, FSH) of anti-rabbit γ-globulin diluted with 0.05 M PBS containing 5% polyethylene glycol (PEG) and incubate at 4°C for 24 h. Centrifugation at 1,700 x g for 30 min at 4°C. Decant the supernatant and swab the extra drop. Count the radioactivity of the precipitate with γ-counter.

B. Testosterone Determination

1. Preparation of testosterone tracer, antiserum and standards

1.1 Testosterone tracer

The stock solution (concentration 10 μCi/ml) was prepared by mixing 250 μCi Testosterone tracer [(^{125}I) testosterone] with toluene: ethanol (9:1) 3 ml. 100 μl of stock solution was pipetted and dissolved in 10 ml of 0.05 M PBS containing 1% BSA. The value of cpm used in this assay was 4,000-5,000 cpm/100 μl.

1.2 Testosterone antiserum

Testosterone antiserum (GDN 250) was dissolved 1:1000 of Testosterone antiserum 1 part: 1000 parts of 0.0025% normal sheep serum). This solution was stored at -80°C as stock solution. When working solution was required, stock solution of testosterone antiserum was added with 0.05 M PBS-0.1% NaN_3-0.05 M EDTA-0.0025% NSS (pH 7.4) and mixed until the final concentration was 1: 300,000.

1.3 Testosterone standard

Testosterone standard was dissolved in methanol. Its concentration was 640 pg/ml; the solution was stored at 4 °C as stock solution. When working solution was required, 100 μl of stock solution of Testosterone standard was added with 900 μl of 0.05 M phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) (pH 7.4). The concentrations of testosterone standard in serial dilution were 0.078, 0.156, 0.313, 0.625, 1.250, 2.500, 5, 10, 20, 40, 80, 160, 320, 640 pg/tube.

2. Procedures of radioimmunoassay for testosterone

2.1 Hormone Extraction

Pipette 100 μl of standard (in 0.05 M phosphate buffered saline (PBS) containing 1% BSA) or 100 μl of sample into 10 x 75 mm disposable glass tube, and

then add 300 μl of tri-distilled water. Add 2 ml of anhydrous ethyl ether and mixed for 3 min. The aqueous phase was frozen in dry ice bath with ethanol. Then the ether phase was decanted to another tube. The ether phase was dried up at 50°C. The wall of the tube was rinsed with 0.5 ml of ether and the drying step was repeated.

2.2 Testosterone Assay

Pipette 100 μl of 0.05 M PBS containing 1% BSA into the extracted test tubes. Pipette 100 μl of anti- testosterone (GDN 250; 1: 300,000) in 0.05 M PBS containing 0.25% normal sheep serum (NSS) and 0.05 M EDTA) into the extracted test tubes. Pipette 100 μl of ^{125}I-labeled testosterone (in 0.05 M PBS containing 1% BSA) into each tube and incubated at 4°C for 24 h. Pipette 100 μl of anti-sheep γ-globulin diluted with 0.05 M PBS containing 5% PEG. The extracted test tubes were incubated at 4°C for 24 h. The extracted test tubes were centrifuged at 1,700 x g at 4°C for 30 min. Decant the supernatant and swab the extra drops. Count the radioactivity of the precipitate with a γ-counter.

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