國立臺灣大學生物資源暨農學院動物科學技術學系

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College of Bio-resources and Agriculture

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Master Thesis

Kisspeptin-10 於輔助生殖技術中取代 PMSG 與 hCG 之可行性

The feasibility of replacing PMSG/hCG with kisspeptin-10 in assisted reproduction technology

陳沛祺

Pei-Chi Chen

指導教授：邱智賢 博士 / 鍾德憲 博士

Advisor: Chih-Hsien Chiu, Ph.D. / De-Shien Jong, Ph.D.

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Declaration of Originality

This is to certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

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中文摘要

由下視丘、腦垂腺、性腺構成的 HPG 軸是控制動物的生殖系統的重要中樞，下視丘會藉由分泌促性腺激素釋放激素(GnRH)刺激腦垂腺分泌促黃體生成素(LH)與促濾泡成熟激素(FSH)，腦垂腺分泌的激素會進一步影響下游性腺的發育與成熟，調控包括雌性與雄性的性內泌素生合成與配子的形成等生理功能。而輔助生殖技術(ART)就是透過調控 HPG 軸操作動物的胚或是配子的一項技術。畜牧業經常使用外源性激性腺素使動物發情同期化或是誘導排卵是 ART 的範疇之一。業界經常使用的外源性激性腺素除了 HPG 軸上的內泌素 GnRH、LH、FSH 與它們的類似物之外，孕馬血清激性腺素(PMSG/eCG)與人類絨毛膜激性腺素(hCG)也是經常使用的激素。PMSG 與 hCG 被廣泛用於取代 FSH 與 LH 的使用，除了前者具有後者類似的生物活性之外，也有更長的體內半衰期，使用上更加方便與經濟，而因此受到業界的喜愛。然而，多次接受 PMSG 或 hCG 處理的動物會因為體內受到外源性內泌的素刺激而產生抗體，繼而對處理不敏感或無反應。此現象在豬、牛、羊中都有紀錄，顯示利用 PMSG 與 hCG 調控動物生殖週期的方法雖然有效但是並不永續。因此，發展出一套有別於傳統內泌素刺激法又能夠延長動物使用年限的生殖週期調控技術是目前重要的研究方向。

另外一方面，負責調控動物 HPG 軸上游的一群胜肽 kisspeptin 近年來受到注意，其中最小的生物活性片段 kisspeptin-10 是由十個氨基酸組成，研究發現 kisspeptin 能夠影響 GnRH 與 LH/FSH 的分泌，對於動物生殖系統的功能與發育具有重要的角色。此外，kisspeptin 在物種之間的相似度非常高，顯示 kisspeptin 在跨物種間的應用有不容易產生抗體的優點。

為了探究 kisspeptin-10 能否於動物輔助生殖技術中取代 PMSG 與 hCG 的使用，我們以小鼠的超級排卵為試驗模型。利用 9 至 12 週齡的 ICR 母鼠進行取代 PMSG

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或是 hCG 的試驗。在取代 PMSG 的試驗中，以迷你幫浦作為緩釋媒介，給予動物三種不同濃度的 kisspeptin-10 (1, 10, 100 μg/100 μl) ；而取代 hCG 的試驗中則是透過單劑腹腔注射給予不同濃度之 kisspeptin-10。紀錄不同處理之間的動物排卵率與排卵數，卵巢組織取下後透過石蠟包埋切片觀察不同處理組之間的差異。在取代 PMSG 的試驗中，經過 kisspeptin-10 處理的小鼠排卵率達到 83%；而控制組與正控制組的排卵率分別是 50%與 100%。傳統的超級排卵方法得到最多的排卵數，而 kisspeptin-10 與控制組得到的平均排卵數相似。在取代 hCG 的試驗中，控制組的排卵率為 20%，而低劑量的 kisspeptin-10 (1 μg/100 μl) 排卵率為 25%，中高劑量的 kisspeptin-10 (10, 100 μg/100 μl) 沒有任何一隻動物排卵。為了更加確認單劑 kisspeptin 對於排卵的影響，我們延長了收卵的時間，從本來打完 hCG 之後 13~14 小時收卵改成 18~20 小時收卵。結果顯示控制組與 kisspeptin-10 高劑量組的小鼠排卵率相同，而排卵個數雖然 kisspeptn-10 的組別較少，但是於統計上與控制組沒有顯著差異。

綜合以上，此研究證明了經由皮下長時間給予 kisspeptin-10 能夠刺激小鼠的卵巢濾泡發育，發情同期化，而誘發排卵的數量又與一般正常週期的小鼠相似。單劑腹腔注射的 kisspeptin-10 相較於 hCG 則有延長排卵的效果，此部分的詳細機制仍需更多試驗得知。而透過皮下給予 kisspeptin-10 長時間刺激動物能有效地影響濾泡發育，將來可應用於動物的生殖週期調控以及生產管理。

關鍵字：kisspeptin-10, 輔助生殖技術，孕馬血清激性腺素/人類絨毛膜激性腺素

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Abstract

Animal reproductive system is controlled by the hypothalamic-pituitary-gonadal axis (HPG axis). The hypothalamus secretes gonadotropin-releasing hormone (GnRH) and regulates the anterior pituitary gland to produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The gonads produce estrogen and testosterone in response to FSH/LH. Assisted Reproductive Technologies (ART), which refers to the various procedures and techniques involving the handling of animals' sperm, oocytes and embryos. ART in livestock widely use exogenous gonadotropins to alter the activity of HPG axis, include GnRH, LH, FSH, pregnant mare serum gonadotropin (PMSG/eCG), human chorionic gonadotropin (hCG) and their artificial analogs. Among these hormones, PMSG and hCG are the preferred alternatives than FSH and LH with longer half-lives and lower cost. However, animals received multiple PMSG/hCG treatment would become insensitive in response. The development of antibodies against PMSG/hCG was reported in heifers, gilts, sheep and goats, which implies that the PMSG/hCG treatment in animal industry may be useful in time but not sustainable. Therefore, to develop alternatives to replace conventional protocol that could prolong the use of animal while sustaining their reproductive ability is relatively important.

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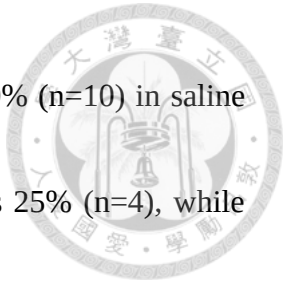
On the other hand, kisspeptin-10, which is the smallest functional kisspeptin peptides made up of ten amino acids, is a potential candidate to replace the conventional hormones with its role in reproductive physiology - the gatekeeper of gonadotropins. Kisspeptin stimulates the secretion of LH/FSH by regulating the activity of GnRH neurons. Moreover, kisspeptin is highly conserved among species, which reduce the chance of antibody development.

To investigate the feasibility of using kisspeptin-10 in replacing PMSG/hCG, we modified the conventional superovulation protocol on ICR mice. Female ICR mice between 9~12 weeks of age were used, and given different doses (1, 10, 100 μg/100 μl) of kisspeptin-10 through time-released Alzet® micro-osmotic pump (1 μl/hr) to substitute for PMSG; and a single injection of kisspeptin-10 at different level (1, 10, 100 μg/100 μl) to displace hCG. The percentage of ovulated mice and the number of ovulated oocytes were counted, and the ovaries were collected for further tissue examination. In the trial of replacing PMSG, the ovulation rate of kisspeptin-10 administrated mice was 83% (n=6) while the untreated group and the conventional protocol group were 50% and 100% (n=6). The conventional protocol group had highest average retrieved oocyte numbers, while the others were similar to the untreated

doi:10.6342/NTU201703852



control group. The trial of replacing hCG, the ovulation rate was 20% (n=10) in saline control group, and the low dose of kisspeptin-10 (1 μg/100 μl) was 25% (n=4), while the medium dose and high dose (10, 100 μg/100 μl) groups were 0% (n=4) and 0% (n=8). The conventional protocol group was 100% (n=8). In another trial, to confirm the effect of single dose kisspeptin-10 to ovulation, the oocyte retrieval time was prolonged from 13-14 hour to 18-20 hour. The ovulation rate and ovulated oocyte was similar between saline control group versus kisspeptin-10 (100 μg/100 μl) group (33%, n=6).

In conclusion, our study demonstrated the effect of long-term peripheral kisspeptin-10 administration on mice reproduction, which has a follicular stimulated potential and synchronizes the estrous cycle with normally ovulated oocyte number, while a single dose of kisspeptin-10 at higher levels seems to prolong the ovulation. The results indicate that the long-term stimulation of kisspeptin-10 could affect the follicular development, which could be a new strategy in livestock reproduction management after some further investigation.

Key words: kisspeptin-10, ART, PMSG/hCG

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Table of Contents

doi:10.6342/NTU201703852

doi:10.6342/NTU201703852

List of Figures and Tables

doi:10.6342/NTU201703852

1. Introduction

The foundation of female reproductive physiology has been focused on the development of a healthy follicle capable of responding to the appropriate hormonal stimuli to grow, after ovulate an ovum, and subsequently develop into a corpus luteum. Since the H-P-G axis is known to control the female estrous cycle, exogenous gonadotropins have been used to alter the female reproductive cycle. The PMSG and hCG, which mimic FSH and LH, are the most used exogenous gonadotropins in mammal animal field. The advantage of PMSG and hCG is that the long half-life and strong stimuli make the reproduction management more efficient, while the drawback of PMSG and hCG are over-stimulating the ovary and inducing the development of antibodies. The irreversible harmful effect is done to female reproductive system via using these exogenous gonadotropins (Gonzalez *et al.*, 1994).

Kisspeptin-10 is the smallest bioactive form of kisspeptins. Kisspeptin system regulates the H-P-G axis from the upstream and the administration of kisspeptin both centrally and peripherally can alter the gonadotropins secretion. Most species share the same kisspeptin-10 sequence, which provide an alternative for reproduction management (Okamura *et al.*, 2013). The study aims to evaluate the feasibility of using

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exogenous kisspeptin to replace the use of PMSG and hCG, which could provide a more sustainable practice in ART.

doi:10.6342/NTU201703852

2. Literature Review

2.1 Assisted Reproductive Technology

Assisted Reproductive Technologies (ART) refers to the various procedures and techniques involving the laboratory handling of human or animals' sperm, oocytes and embryos such as artificial insemination (AI), in vitro fertilization (IVF), animal cloning, cytoplasmic transfer, hormone treatment, zygote intrafallopian transfer (ZIFT), cryopreservation/vitrification of sperm/oocytes/embryos, intracytoplasmic injection (ICSI), … etc. (Hafez, 2015) These techniques provide a powerful tool from animal-based research to clinical infertility treatment. In biomedical research, Laboratory mice (*Mus musculus*) are the most frequently used animals for biomedical research with a well-defined genetic background and relatively short reproductive cycle, which are easy to establish animal models for specific diseases. In agriculture practices, ART are used to promote livestock production and management. Animals with genetic merit could be preserved and multiplied; estrus synchronization gives an alternative to better farm management. In clinical application, human infertility is the main target of these technologies.

2.1.1 Exogenous gonadotropins

doi:10.6342/NTU201703852

Exogenous gonadotropins are widely used in ART, such as gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), follicle stimulating hormone (FSH), pregnant mare serum gonadotropin (PMSG), human chorionic gonadotropin (hCG) and their artificial analogs. Traditional ovary stimulation protocols frequently used LH and FSH, which usually extracted from animal pituitaries. However, the half-life of extracted LH and FSH are relatively short (~5 hour), multiple treatments are required to have a successful stimulation. Among these gonadotropins, PMSG and hCG are two of the most frequently used gonadotropins in animal ART, which have longer half-live (>50 hour) and are used to replace for FSH and LH, respectively (Mapletoft *et al.*, 2002).

PMSG, or equine chorionic gonadotropin (eCG), is a complex glycoprotein produced from the endometrial cups of pregnant mares. Cole and Hart (1930) found that PMSG has potent gonadotropic activity, both on follicle stimulating (FSH-like) and luteinizing (LH-like) bioactivity. Since then, the PMSG had been extracted for use in anovulatory women. In veterinary medicine, it was recommended for the stimulation of ovarian function and activity, improvement of fertility in estrus-synchronized animals and the induction of superovulation (Cole and Hart, 1930).

doi:10.6342/NTU201703852

On the other hand, human chorionic gonadotrophin (hCG), was first described in 1943, which was found to be secreted from trophoblastic cells (Jones *et al.*, 1943). The mice were administrated urine from pregnant women and the development of follicular cysts and hemorrhagic follicles were observed, which was recognized as the first pregnancy test (Zondek and Aschheim, 1927). Because of the half-life of hCG is significantly longer than LH, hCG is widely used for inducing ovulation.

The advantages of using PMSG and hCG as a reproduction treatment are:

- higher doe receptivity rate,
- higher conception rate,
- larger litter size,
- effects are stronger when body conditions are weaker (Szendro *et al.*, 2012).

2.1.2 ART using PMSG/hCG

In 1941, the concept of the two-step protocol was developed: ovarian stimulation using gonadotrophins and the induction of ovulation using hCG. PMSG was used in this protocol in amenorrhoeic women with good results: about 83% of severely amenorrhoeic women whom received either PMSG or a pituitary extract from pigs and sheep for follicle stimulation,while hCG for the induction of ovulation, led to a

doi:10.6342/NTU201703852

successfully menstruation. These results suggested the presence of PMSG/hCG stimulated mature follicle development (Mazer, 1946).

Superovulation is one of the ARTs that aims to enhance the yield of viable embryos by increasing the ovulation rate. It is a fundamental practice for producing transgenic mice or embryology research. The method for obtaining mature ovulated mice oocytes was first described in 1957 (Fowler and Edwards, 1957). Since then, the PMSG/hCG treatment is the most recognized method in rodent superovulation. This protocol was well established and has been used for over fifty years. Superovulation is also used in livestock industries to preserve and multiply animals with preferred genetic background or productivity merits. Such as dairy cows (Bevers and Dieleman, 1987), sheep (Martemucci *et al*., 1995) and goats (Pintado *et al.*, 1998).

The value of estrus synchronization is vital in dam reproductive management, as the duration of both estrous cycle and estrus is variable and estrus detection cannot be accomplished without a sire. It is an important ART in farm reproductive management which alters a group of dams' estrous cycle via exogenous gonadotropins or other hormones to become estrus or ovulate during certain range of time. The procedure provides a more feasible and convenient platform for artificial insemination or

doi:10.6342/NTU201703852

oocyte/embryo collection (Jainudeen *et al*., 2000). In swine, PMSG combine with hCG is used for estrus induction or inducing ovulation. In sheep and goats, PMSG combined with progesterone intra-vaginal device (controlled internal drug-releasing dispenser, CIDR) is a common strategy for estrus synchronization and induce estrus (Kusina *et al*., 2000). Moreover, in small ruminants, the use of CIDR and PMSG are not only for estrus synchronization, but also for anestrus season breeding management, resuming the reproductive activity (Rahman *et al.*, 2008).

2.1.3 Drawback of the use of PMSG/hCG

Since PMSG was found to have gonadotropic activity in different species, it was widely used in 1930s, especially for clinical treatment to women with abnormal menstrual cycle. In 1940s, however, women whom receive the treatment more than twice had become insensitive to the treatment. The so called 'antihormones' were developed (Ostergaard, 1942; Zondek and Sulman, 1942). This put the use of PMSG to the end in clinical (Ludwig *et al*., 2011).

Same situation happened in livestock industry. The resistance of PMSG also found in heifers, gilts, goats (Pintado *et al*., 1998) and sheep (Martemucci *et al*., 1995) which received multiple administration. To solve the problem, antiserum was used to eliminate

doi:10.6342/NTU201703852

the immune response of treated animal. Though the modified protocol yields more oocytes and higher superovulation response, and more feasible in estrus synchronization, the time course of the protocol is species dependent and variation is large between individuals (Bodin *et al.*, 1997).

PMSG also increased undesired elevation of estradiol during the treatment. Owing to the long half-life of PMSG, the follicles are still being stimulated after ovulation occurred, which leads to high estradiol levels. High level of estradiol during early luteal phase will have deleterious effect on the oviduct and decrease fertility. The increased numbers of large follicles at the time of ova and/or embryo collection can also affects collection efficiency and embryo quality (Gonzalez *et al.*, 1994).

Although other modified PHSG/hCG protocol had been developed, some research has shown that repeated administration of PMSG/hCG may induce strong oxidative stress and damage to the female reproductive tract (Park *et al.*, 2015), and promotes the apoptosis of ovarian cells (Dong *et al.*, 2014).

The immune response of hCG repeated administration was also reported in dairy cows (Giordano *et al.*, 2012), which implies that the PMSG/hCG treatment may be useful in time but not sustainable. This may not be a critical drawback in laboratory,

doi:10.6342/NTU201703852

since most of the superovulated mice are euthanized after the oocytes were collected. But when it comes to the reproductive management of the livestock, the sustainability of this exogenous gonadotropin treatment is questioned. Therefore, to develop alternatives to replace conventional protocol that could prolong the use of animal while sustain their reproductive ability is relatively important.

To sum up, PMSG/hCG treatment may have some negative effects such as:

- ineffective with non-receptive does,
- produce anti-PMSG and anti-hCG antibody following frequent and/or high dosage treatment,
- lower conception rate after frequent use,
- higher mortality after birth,
- most of consumers are concerning about the use of hormones in animal production.

(Szendro *et al.*, 2012)

2.2 Kisspeptin in reproduction

The reproduction is an essential component to all species. Fertility in animals is initiated at puberty by the pulsatile secretion of gonadotropin releasing hormone (GnRH)

doi:10.6342/NTU201703852

from hypothalamus. The GnRH is released into the hypophyseal portal blood system and affect the anterior pituitary to stimulate the secretion of the LH and FSH. The gonadotropins act on the gonads to stimulate sexual maturation and gametogenesis. The gonads produce sex steroid hormones which are essential for gametogenesis and the maturation of reproduction organs, and provide hormonal feedback to control the release of GnRH and gonadotrophin under different reproductive status. This loop is the well-known hypothalamic-pituitary-gonad axis (HPG axis), the central concept of animal reproduction. Although GnRH neurons are thought to be the critical component of the HPG axis, it was found that kisspeptin system plays a vital role upstream and regulate GnRH release. Kisspeptin integrate central and peripheral signals and affect both animal reproduction and energy metabolism (Bond and Smith, 2014). It is considered the gatekeeper of animal reproduction (Wahab *et al.*, 2013).

Kisspeptin, which was called metastin, was first found in cancer research that identified as a breast cancer metastasis suppressor (Lee *et al.*, 1996). Kisspeptins are an overlapping set of short chain peptides encoded by the Kiss1 gene. The peptides were originally isolated from human placenta (Kotani *et al.*, 2001). The longest kisspeptin contains 54 amino acids in length (Kp54), shorter forms have also been identified,

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including Kp10, Kp13, and Kp14. These shorter kisspeptins may be the cleavaged products, but they still remain full bioactivity. Kisspeptins are highly conserved between species, the shorter the peptide is, the more similar they are. The receptor of kisspeptin, GPR54, a kind of G protein-coupled receptor, was first found in 1999. It was named new orphan G protein-coupled receptor due to the ligand to this receptor wasn't identified until 2001 (Gottsch *et al.,* 2009).

Kisspeptin expression was identified at high levels in the placenta, and has been observed in the testis, ovary, pancreas, and small intestine (Ohtaki *et al.,* 2001; Gaytan *et al.,* 2009). Central expression of kisspeptin and its receptor have been discovered in two major neuronal populations. In rodents, in situ hybridization and immunohistochemistry have been used to map kisspeptin neurons to two regions of the hypothalamus, the arcuate nucleus (ARC) and in a periventricular continuum of cells within the rostral part of the third ventricle, including the anteroventral periventricular nucleus (AVPV) (Gottsch *et al.,* 2004; Clarkson *et al.,* 2006, 2008, 2009).

2.2.1 Kisspeptin regulates HPG-axis

Kiss-1 and GPR54 are found in many organs in animal, while the functional aspect in animal physiology has not been fully investigated. In animal reproduction, kisspeptin

doi:10.6342/NTU201703852

is secreted by a group of neurons called kisspeptin neuron, recognized as the upstream regulator of HPG-axis (Kotani *et al.,* 2001; Muir *et al.,* 2001; Funes *et al*., 2003). The distribution of kisspeptin neurons in the hypothalamus varies between species (Colledge, 2009). Humans (Rometo *et al.,* 2007), rhesus monkeys (Shahab *et al.,* 2005), and sheep (Franceschini *et al.,* 2006; Smith *et al.,* 2007) have proportionally more kisspeptin neurons in the ARC than in the AVPV region. Kisspeptin expression increases in both regions during pubertal development in rodents (Navarro *et al.,* 2004; Clarkson et al., 2006; Takase *et al.,* 2009) and monkeys (Shahab *et al.,* 2005). Research in mammals has proven that kisspeptin can bind to GPR54 and stimulate the GnRH neurons secret GnRH and regulate the downstream hormone both in female and male (Gottsch *et al.,* 2004; Irwig *et al.,* 2004; Aparicio, 2005). In rodents, however, the kisspeptin neurons of the AVPV appear to be sexually dimorphic, with many more neurons in females than in males (Clarkson *et al.,* 2006; Kauffman *et al.,* 3007). Some evidences also support the possibility of sexually dimorphic kisspeptin neuron populations in the rostral periventricular area of the third ventricle (RP3V) and infundibulum of humans (Hrabovszky *et al*., 2010, 2011). It has been observed that the increase in kisspeptin

doi:10.6342/NTU201703852

expression within the RP3V during pubertal development was dependent upon estradiol in female mice (Clarkson *et al.*, 2009; Bakker *et al.*, 2010).

GnRH neurons express the kisspeptin receptor (d'Anglemont *et al.*, 2008; Herbison et al., 2010). When hypothalamus is stimulated by kisspeptin neurons, it will release gonadotropin release hormone (GnRH) and then the pituitary secretes luteinizing hormone (LH) and follicle stimulating hormone (FSH) in response both in vitro and in vivo studies (Irwig *et al.*, 2004; Liu *et al.*, 2008; Novaira *et al.*, 2009), while the effect was inhibited by the administration of GnRH antagonists (Shahab *et al.*, 2005). The kisspeptin neurons along with HPG-axis controls the development of gonads and the onset of puberty, and also coordinate with gonads to regulate the estrous cycle in female animals.

It has been proven that kisspeptin can stimulate hypothalamus to secrete GnRH through central or peripheral administration (Gottsch et al., 2004; Navarro et al., 2004; Dhillo et al., 2005; Messager et al., 2005; Navarro et al., 2005; Shahab et al., 2005). The LH and FSH raised when a very low dose of kisspeptin (1 fmol) is given to mice through lateral ventricle (Gottsch *et al.*, 2004). Other research on rat (Matsui *et al.*, 2004), sheep (Messager *et al*., 2005), monkeys (Shahab *et al*., 2005), and human (Dhillo

doi:10.6342/NTU201703852

et al., 2005) also showed similar results. Expression of the kisspeptin receptor gene has been observed in both the ARC and AVPV. Kisspeptin neurons stimulate the cell bodies of GnRH neurons in the preoptic area, and to the median eminence, which is close to GnRH nerve endings (Gottsch *et al*., 2004; Clarkson *et al.*, 2006; Uenoyama *et al.*, 2011).

Taken together, these data suggested that kisspeptin stimulates GnRH neurons in the hypothalamus to release GnRH into the hypothalamic-pituitary portal circulation, causing the release of gonadotropins from the anterior pituitary (Messager *et al.*, 2005).

2.2.2 Kisspeptin triggers puberty and maintain fertility

In 2003, scientists discovered that kisspeptin-GPR54 system is vital to the development of animal reproductive system and sexual maturation. Animals which have mutant GPR54 receptor will develop idiopathic hypogonadotropic hypogonadism (IHH) and fail to reach puberty (de Roux *et al.*, 2003; Seminara *et al.*, 2003). The IHH phenotype has also been observed in patients with heterozygous kisspeptin receptor mutations (Chan *et al.*, 2011), suggesting a vital role of kisspeptin in puberty. Moreover, an inactivating mutation in the kisspeptin gene in humans with absent progression of puberty has also been reported (Topaloglu *et al.*, 2012).

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The knockout of GPR54 receptor would lead to the loss of fertility in mice. The use of knockout mouse models has allowed more advanced study on the exact mechanism and function of kisspeptin in sexual maturation. In 2003, first study showed that kisspeptin receptor null mice displayed hypogonadotropic hypogonadism (HH), might develop due to low levels of circulating gonadotrophin hormones, with small testes in male mice and a delay in vaginal opening and an absence of follicular maturation in female mice. The administration of exogenous GnRH corrected the HH phenotype, which was consistent with the concept that kisspeptin acts by stimulating endogenous GnRH (Funes *et al.*, 2003; Seminara *et al.*, 2003).

Kisspeptin-expressing neurons in the AVPV of mice are only detectable from postnatal day 25, with peak adult levels by the onset of puberty at day 31 (Clarkson *et al.*, 2006). A research in 2004 demonstrated the central injections of kisspeptin to mice from postnatal day 26 to day 31 induced advanced vaginal opening, increased uterine weight and raised plasma LH and estradiol levels relative to the controls. It was the first research that suggested the role of kisspeptin in the animal puberty (Navarro *et al.*, 2004). Four years later, Teles et al. (2008) identified an activating autosomal dominant mutation in the kisspeptin receptor gene in a girl with advanced puberty. These studies

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has paved the way for many other investigation about the role of kisspeptin in the puberty.

Kisspeptin may also regulate seasonal reproduction in certain species. Increased hypothalamic kisspeptin expression has been reported in Syrian hamsters during long day conditions, associated with increased sexual activity (Revel *et al*., 2006). Revel et al. (2007) observed that the administration of kisspeptin-10 to Syrian hamsters under photo-inhibitory conditions could restore testicular weight and activity, and also the reproductive function. Sheep are also known to be seasonal breeders, with increased reproductive activity during short photoperiod. ARC kisspeptin expression increased in ewes during short day conditions, but no change in kisspeptin expression levels in AVPV (Clarke et al., 2009). Conversely, during long day periods kisspeptin expression in the ARC of ewes is reduced (Smith *et al.*, 2007). Recently, it has been suggested that GnRH (and LH) responses to kisspeptin are greater in anestrus ewes compared with luteal phase ewes. In addition, kisspeptin receptor expression on GnRH neurons was greater during the non-breeding season compared with the breeding season. It is thought that GnRH increased the sensitivity to kisspeptin for the re-initiation of the breeding season (Li *et al.*, 2012). A recent study examined expression of kisspeptin in Syrian

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hamsters, which are long-day breeders. They observed that the expression of kisspeptin was down-regulated in the ARC under a short photoperiod (Barzten *et al*., 2014).

To sum up, kisspeptin-GPR54 system is important to stimulate the release of gonadotropins during puberty and sustain the fertility during adulthood. Also, it regulates the estrous cycle both in seasonal breeder and non-seasonal breeder.

2.2.3 The feedback loop of steroid hormones

It is well known that steroid hormones produced by the gonads send feedback signaling to the hypothalamus to regulate GnRH production and the release. Estrogen is known to exert its positive feedback via centrally located estrogen receptors (ERs) to induce the LH surge (Couse *et al.*, 2003; Wintermantel *et al.*, 2006). However, GnRH neurons lack the ERs (Herbison *et al.*, 1992), suggesting there is certain neuronal pathway between GnRH neurons and ERs. Key work by Smith investigated the potential role of kisspeptin in mediating the estrogen-induced LH surge (Smith *et al.*, 2006). They observed that kisspeptin expression in the AVPV of rats was highest during the evening of proestrus, whereas expression levels in the ARC were at their lowest during this time. In ovariectomized rats, kisspeptin expression was increased in the AVPV at the time of an exogenous estrogen and progesterone-induced LH surge,

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whereas kisspeptin expression in the ARC was at its lowest during this time. The research implied that the central expression of kisspeptin is related to animal estrous cycle and is regulated by estrogen. Ovariectomy may abolish the kisspeptin-induced GnRH release in pubertal monkeys, and estradiol replacement may result in partial recovery of kisspeptin-induced GnRH release (Guerriero *et al.*, 2012). A research on acyclic ewes also found that the systemic delivery of kisspeptin induced LH surges by activating estradiol positive feedback on gonadotropin secretion (Sebert *et al.*, 2010). These data suggest that kisspeptin requires estradiol to stimulate GnRH secretion.

Furthermore, kisspeptin neurons in the AVPV co-express the immediate early gene Fos, which is an indicator of LH surge (Hoffman *et al.*, 1990), at the time of the proestrus, whereas minimal Fos expression was observed on diestrous. In contrast, kisspeptin neurons in the ARC did not express Fos during the LH surge or on diestrous. Lastly, most kisspeptin neurons in both the AVPV and ARC express the ER. These data suggest that kisspeptin neurons in the AVPV play a role in mediating estrogen signaling to generate the preovulatory LH surge (Smith *et al.*, 2006).

A number of other studies have investigated the role of kisspeptin signaling in the LH surge. Exogenous kisspeptin administration has been observed to potently induce

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LH secretion resulting in ovulation in rats (Matsui *et al.*, 2004; Navarro *et al.*, 2005). Furthermore, the estrogen-induced preovulatory surge is inhibited by the administration of anti-kisspeptin antibodies in rats (Kinoshita *et al.*, 2005; Adachi *et al.*, 2007). Clarkson et al. (2008) observed that, in Kiss1 knockout mouse models, kisspeptin receptor signaling was critical for the LH surge and subsequent ovulation. In contrast, kisspeptin receptor knockout mice underwent an estogen-induced LH surge, suggesting that there might be several different pathways to alter the process of LH surge (Dungan *et al.*, 2007).

Tomikawa group examined the epigenetic regulation of kisspeptin gene expression mediating estrogen-positive feedback action in mice (Tomikawa *et al.*, 2012). They observed that the histone of the kisspeptin gene locus in the AVPV was highly acetylated, and the ER was highly recruited at the region by estrogen, whereas the same locus in the ARC showed histone deacetylation in response to estrogen. This suggested that epigenetic regulation of kisspeptin may regulate kisspeptin expression in the AVPV in response to estrogen, and underlies the estrogen positive feedback resulting in the LH surge.

2.2.4 The local effects of Kisspeptin in gonads

doi:10.6342/NTU201703852

While the central effects of kisspeptin are increasingly well investigated, there are some hints that direct gonadal effects of kisspeptin may also exist. In 2004, Terao first observed the expression of the genes encoding kisspeptin and its receptor in rat ovaries (Terao *et al.*, 2004), which has also been found in primate and human ovaries (Cejudo *et al.,* 2012). Futhermore, it was observed that ovarian expression of kisspeptin was cycle dependent in rats (Castellano *et al.,* 2006).

Evidence of the effects of kisspeptin locally on ovaries in mice has been investigated, and it was independent of its central effects via gonadotrophins. Gaytan et al. (2014) observed that both kisspeptin receptor null mice and haplo-insufficient mice had premature ovarian failure (POF), and it was related to ovarian kisspeptin receptor expression, while the levels of circulating gonadotrophins remained normal. The research implied a direct interaction between kisspeptin and the ovaries (Gaytan *et al.*, 2014). Furthermore, another research demonstrated the neurotrophin signaling via the ovarian neurotrophic receptor tyrosine kinase 2(NTRK2) receptor, which is essential for oocyte maturation. It was found that NTRK2 is dependent upon kisspeptin receptor signaling using Ntrk2 knockout mouse models. They suggested that both signaling

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pathways were required for oocyte survival and follicular integrity in the adult ovary through the communication between granulosa cells and oocytes (Dorfman *et al*., 2014).

The kisspeptin and its receptor are not only locally affecting female reproductive organ but also in male. The genes encoding kisspeptin and its receptor are expressed in both human and rodent testes (Funes *et al.*, 2003; Terao *et al.*, 2004). Kisspeptin administration enhanced hCG-stimulated testosterone release in GnRH receptor antagonist treated male monkeys, but had no effect when kisspeptin administrated alone in GnRH receptor antagonist treated monkeys. The research implied that kisspeptin may enhance the effect of hCG on testosterone release through a novel pathway (Irfan *et al.*, 2014).

Also, kisspeptin and its receptor were deceted in human spermatozoa, and the exposure to kisspeptin resulted in a rise in intra-cellular calcium, with increasing sperm motility (Pinto *et al*, 2012). Furthermore, Hsu et al. (2014) suggested that kisspeptin modulates the fertilization capacity of mouse spermatozoa by promoting capacitation, and kisspeptin antagonist, peptide 234, reduced fertilization rates of spermatozoa. The significance of these results is that aside with the central regulation of kisspeptin, it may act peripherally to regulate gonadal function in both males and females.

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2.2.5 Kisspeptin's prospect in ART

As mentioned previously, kisspeptin-10 is the smallest functional kisspeptin peptides made up with ten amino acids; and it is highly conserved among species (Okamura *et al.*, 2013). Since kisspeptin-10 is a relatively small peptide and many animals share the same amino acid sequence, there is much lower chances that the recipients would produce antibodies when peptide given exogenously. This merit implies the sustainability of using kisspeptin-10 in animal reproduction management could be better compared to conventional methods using PMSG and hCG.

Compared to other GnRH analogies, kisspeptin-10 can stimulate the secrete of LH without long term exposure suppression (Pinilla *et al.*, 2012). However, the merit can also be a drawback. The half-life of kisspeptin is so short that it has to be given through central nerve system or intravenous perfusion or long-term osmotic pump to reach the threshold to induce a significant effect in vivo, which restrict the application in field and large scale farming system. Nowadays the research of kisspeptin application focus on two alternatives: one is to develop kisspeptin analogs that have a prolong half-life; another is using time-release technic to make sure the peptide can reach a sustainable releasing rate.

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There is plenty research on sheep that if exogenous kisspeptin could resume the estrous cycle in non-breeding season. Given anestrus sheep small amount of kisspeptin through infusion, the GnRH would respond, and several hours later the LH level raised. The phenomenon mimics the change of sex hormone during breeding season, includes the secrete of estrogen, the positive and negative feedback towards LH and FSH (Caraty *et al.,* 2010). Eighty percent of anestrus ewe were detected ovulated when kisspeptin was administrated through intravenously infusion for thirty to forty-eight hours (Caraty *et al.,* 2007). Long-term infusion of kisspeptin can synchronize about eighty percent of anestrus ewe which have surge-like LH secretion infused for 22 hours, and the percentage of ewe with surge-like LH level is related to infusion period (Sebert *et al.*, 2010). The expression of kiss1 receptor on GnRH neuron elevated during non-breeding season, which raise the sensitivity to kisspeptin stimulation (Li *et al.*, 2012). Decourt et al. (2016) use synthesized kisspeptin analog in single intramuscular injection along with progesterone priming successfully synchronize the estrous cycle of ewe during breeding season. The ewe was detected ovulation and lambing after natural mating.

During luteal phase, goats received kisspeptin-10 via iv injection in two-hour intervals showed LH and FSH level change along with the kisspeptin single bolus

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administration. The reaction is relatively low compared to GnRH positive control group (Hashizume *et al.*, 2010). Goto et al. (2014) use artificial compound TAK-683, a kisspeptin-10 analog, to stimulate the goat during estrous cycle; and they found that the goats would have advanced ovulation. When TAK-683 is used on oophorectomized goat, a long-term stimulation given by subcutaneous pump for five days, the LH secretion is suppressed, but retain LH surge activity as control group (Tanaka *et al.*, 2013).

With evidence from rodents and sheep that kisspeptin is a critical stimulus for the LH preovulatory surge, a recent study investigated the potential for kisspeptin to be used in women undergoing in vitro fertilization (IVF) therapy. Jayasena et al. (2014) administered a single injection of kisspeptin-54 at differing doses to women undergoing IVF, following standard recombinant follicle-stimulating hormone and GnRH antagonist therapy. Egg maturation was observed in response to each tested dose of kisspeptin at 36 hours from administration. The mean number of mature eggs per patient increased in a dose-dependent manner. Current practice most commonly uses hCG to trigger egg maturation (Macklon *et al.*, 2006), which acts directly on ovarian LH receptors to stimulate egg maturation. The use of hCG confers a risk of ovarian

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hyperstimulation syndrome (OHSS) due to sustained agonist activity compared with the endogenous LH surge, and a lack of negative feedback control. Thus, by stimulating endogenous GnRH and gonadotropin release at physiological levels, kisspeptin use in IVF therapy may have reduced risk of OHSS, although comparison to existing therapies is required in larger studies.

2.3 Sustained release techniques

Proteins and other bio-therapeutics are currently being developed as potential therapy for many diseases and disorders, and it was predicted that the overall market for biopharmaceuticals will grow further in the coming years (Tang *et al.*, 2004). Proteins and peptides are easily affected by the environment conditions, and are very likely being digested by enzymes in vivo. To sustain the half-life of protein medicines is the key factor to sustain the therapeutic effect. The half-life of protein substances are affected by its own chemical characteristic and the administration pathway. Subcutaneous (SC) administration provides a convenient method for administrating compared with intravenous (IV) administration and has been approved for the clinical treatment of therapeutic proteins like insulin and human growth hormone (Richter *et al.*, 2012). In some cases of proteins with shorter elimination half-lives, SC administration

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has been demonstrated to provide prolonged exposure to these proteins by maintaining high plasma concentrations for longer periods, and cause less stress to the recipient (Hale *et al.,* 2004). Drugs administered by the SC route can reach the systemic circulation either by uptake via blood capillaries or by lymphatic system. Compounds with molecular weights less than or equal to 16 kDa can reach the systemic circulation via blood capillaries. Protein therapeutics with higher molecular weights exhibit limited transport into the blood capillaries and enter the systemic circulation via the lymphatics (Supersaxo *et al.,* 1990). Although SC administration provides longer protein half-life compare to IV administration, small peptides like kisspeptin-10 still degraded quickly in vivo. To solve this problem, the sustained release technique has become a critical strategy both in labs and in fields.

Sustained release technique includes modifying the protein peptides to prolong the half-life or loading the proteins with time-release micro sphere, osmotic pump, and other types of drug form. In the research about the effect of long-term exposure of kisspeptin, the experimental design usually contained osmotic pump to sustain the bioactivity of kisspeptin in an IV infusion manner (Hashizume *et al.,* 2010; Sebert *et al.,* 2010). There was also some research trying to exchange the peptide and modified it to

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extend the half-life (Tanaka *et al.*, 2013; Decourt *et al*., 2016). However, in the case of kisspeptin-10, it is a relatively difficult strategy since there are only ten amino acid made up of kisspeptin-10; and to decide which amino acid to change to prolong the half-life while maintain the bioactivity of the peptide is a time-consuming practice.

2.4 Histology of mouse ovary and follicle development

The female laboratory mice, like most placental mammals, demonstrates intrinsic reproductive cycle, characterized by the regular occurrence of an estrous cycle. The estrous cycle consists of four stages: proestrus, estrus, metoestrus (or dioestrus 1) and dioestrus (or dioestrus 2). Because mice are continuously polyoestrous (i.e., cycle constantly throughout the year) dioestrus is immediately followed by the proestrus phase of the next cycle. Estrous cycle only ceases during pseudo-pregnancy, pregnancy, and lactation, although a fertile postpartum estrus does occur within 24 hours after birth. The first estrous cycle begins within approximately one week after vaginal opening and recurs regularly every 4 or 5 days for a variable proportion of the animal's lifespan, depending on the strain of mice.

The ovary is contained within a thin-walled bursa, which originates from mesovarium that attaches the ovary to the peritoneum. The ovarian bursa is usually

doi:10.6342/NTU201703852

found embedded in fat just caudal to the kidneys. The bursa is lined on both sides by flattened mesothelial cells and has a thin connective tissue core which contains scattered smooth muscle fibers.

The mouse ovary has an outer cortex that contains the follicular structures and Corpus luteum and a central medulla which contains stroma and blood vessels. The division between cortex and medulla is ill defined and, because of the small size of the ovary, the medulla may not be present in every section. The ovarian stroma contains spindle shaped cells interspersed with collagen and supports the follicles and Corpus luteum. The collagen is denser in the area below the surface epithelium and the region is sometimes called the tunica albuginea although it is poorly defined and may be hard to distinguish in mice.

Ovarian follicles are categorized based on the morphological appearance of the associated granulosa cell layers into primordial, primary, secondary, antral and atretic follicles (Myers *et al*., 2004). Primordial follicles tend to be found towards the sub-capsular region of the ovarian cortex and consist of an oocyte surrounded by a single layer of flattened granulosa cells. Polyovular follicles may be seen in young mice but are rare in mature animals (Kent, 1960). The growing oocytes are surrounded by a

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layer of glycoproteins secreted by the oocyte (El-Mestrah *et al*., 2002) called the zona pellucida. In primary follicles the oocyte and zona pellucida is surrounded by a single layer of cuboidal granulosa cells and an outer layer of flattened cells. Secondary follicles have multiple layers of cuboidal granulosa cells in close association with the oocyte, with no antral space. Antral follicles have multiple layers of granulosa cells and a clearly visible fluid-filled antral space (or spaces). The numbers of large antral follicles increase during proestrus and decrease as a result of ovulation during estrus. In large preovulatory antral follicles, the oocyte is separated from a single antral space by a surrounding layer of granulosa cells (the cumulus granulosa), which will be retained with the oocyte when it is released at ovulation.

Most follicles do not reach the point of ovulation and either undergo attrition (primordial follicles) or atresia. Oocytes become atretic as a result of apoptosis and so in early follicular atresia fragmentation of the degenerate oocyte and granulosa cells may be seen.

The appearance of Corpus luteum varies with the stage of the estrous cycle and whether they are from the current or previous cycles. When first forming following ovulation the theca cells (Young and McNeilly, 2010), which make up the Corpus

doi:10.6342/NTU201703852

luteum, have basophilic cytoplasm and are a plump spindle shape, mitoses may be present. The center of a newly formed corpus luteum may have an irregular fluid filled or hemorrhagic cavity. During metoestrus and dioestrus the cells of the corpus luteum become plumper and may start to accumulate fine lipid vacuoles (Greenwald and Rothchild, 1968) reaching maximum size and vacuolation at dioestrus. Fibrous tissue may also be apparent in the center of the corpus luteum at dioestrus filling what was the hemorrhagic cavity (Westwood, 2008). During proestrus the Corpus luteum start to degenerate (with vacuolation of cytoplasm), nuclear fragments and neutrophils may be present. Corpus luteum from up to three previous cycles may be present and are identifiable by being more eosinophilic than the Corpus luteum of the current cycle and becoming paler and smaller with time.

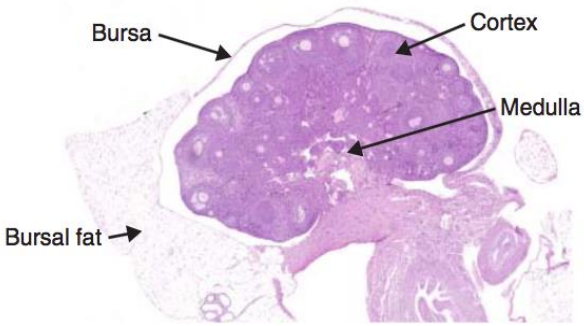

Figure 1. Overview of mouse ovary. (Cheryl, 2014)

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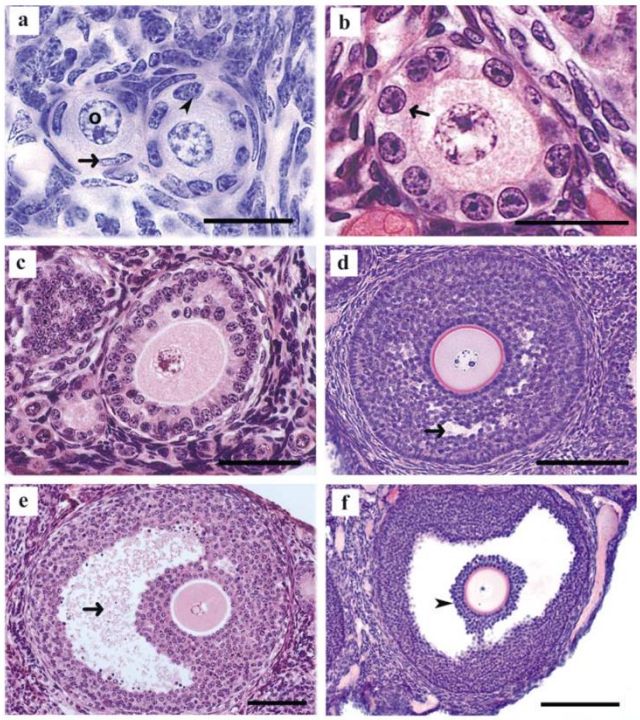


Figure 2. Follicular classification of mice. The classification of follicular stages used for analysis. (a) Primordial follicles were defined as an oocyte (o) surrounded by a layer of squamous (flattened) granulosa cells (arrow). Follicles which possessed predominantly cuboidal granulosa cells (arrow-head) with some squamous granulosa cells were classified as primary. Bar = 20 µm. (b) Primary follicles possessed an oocyte surrounded by a single layer of cuboidal granulosa cells (arrow). Bar = 20 µm. (c) Secondary follicles were surrounded by more than one layer of cuboidal granulosa cells, with no visible antrum. Bar = 50 µm. (d) Early antral follicles have emerging antral spaces (arrow), whilst antral follicles (e) possessed a clearly defined antral space (arrow); bars = 100 µm. (f) Preovulatory follicles were the largest of the follicular types and possessed a defined cumulus granulosa cell layer (arrowhead). Bar = 200 µm. (Westwood, 2008)

doi:10.6342/NTU201703852

3. Materials and Methods

3.1 Animals

Mice. Mature female (9-12 weeks old) ICR mice purchased from BioLASCO Taiwan Co., Ltd were used as oocytes donors. The mice were maintained with a 12:12 h light-dark cycle (lights switched on at 0700 h and off at 1900 h). The room temperature was maintained at 23 ℃ ± 2 ℃. The animals were kept with free access to feed and water. All experimental protocols were approved by the Institutional Animal Care and Use Committee, College of Medicine, National Taiwan University. All procedures followed to the National Institutes of Health Guide for the care and use of laboratory animals.

3.2 Chemicals

Peptides. Murine kisspeptin-10 amide (mKp-10; amino acid sequence: YNWNSFGLRY – NH_2) was purchased from a commercial peptide synthesizer (Kelowna).

Pregnant mare serum gonadotropin. PMSG sterile filtered white lyophilized (freeze-dried) powder was purchased from ProSpec®.

doi:10.6342/NTU201703852

Chorionic gonadotropin human. hCG lyophilized powder purchased from Sigma® was used.

Hyaluronidase. 0.1% of hyaluronidase is used for the removal of cumulus cell. Incubate cumulus-oocyte complexes (COCs) retrieved from the oviduct with hyaluronidase solution for few minutes. Hyaluronidase powder was purchased from Sigma® and was dissolved in distilled deionized water in 1% as stock solution.

3.3 Time-Release of kisspeptin-10

ALZET® micro-osmotic mini pump were used to sustained the release of kisspeptin-10 subcutaneously. Product #1003D were purchased, which can release for 3 days and release at a rate of 1 µl per hour.

3.4 Experimental Design

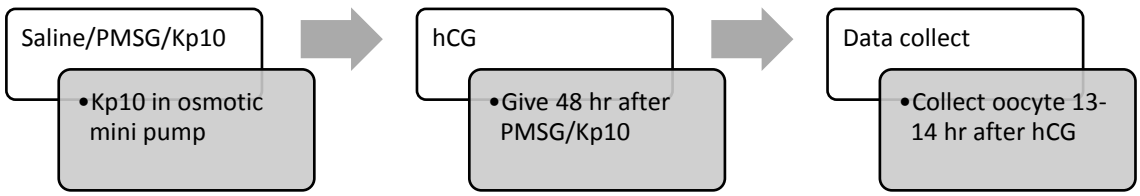
3.4.1 PMSG replacement trial

Saline control protocol. A blank osmotic mini-pump loading with saline was given s.c., delivering 1µl per hour to each female. The duration of the mini-pump was 72 hours and hCG (10 IU) was given by i.p. 48 hours after the pump implant surgery was done. The mice were sacrificed 13-14 hour after the hCG treatment by cervical dislocation, oviducts were removed and ova were recovered.

doi:10.6342/NTU201703852

Kisspeptin protocol I~III. Three different levels of kisspeptin-10 (1 μg/100 μl, 10 μg/100 μl and 100 μg/100 μl) were administrated as a continuous release via an osmotic mini-pump implanted s.c., delivering 1μl per hour to each female. The duration of the mini-pump was 72 hours and hCG (10 IU) 0.1 ml was given by i.p. 48 hours after the pump implant surgery was done. The mice were sacrificed 13-14 hour after the hCG treatment by cervical dislocation, oviducts were removed and ova were recovered.

Conventional protocol. Mice received PMSG (eCG, 5 IU) by i.p. injection at day 1 and given hCG (10 IU) by i.p. injection 48 hours later (day3). The mice were sacrificed 13-14 hour after the hCG treatment by cervical dislocation, oviducts were removed and ova were recovered.



3.4.2 hCG replacement trial

Saline control protocol. Mice received PMSG (eCG, 5 IU) by i.p. injection at day 1 and given 100 μl of saline by i.p. injection 48 hours later (day3). The mice were

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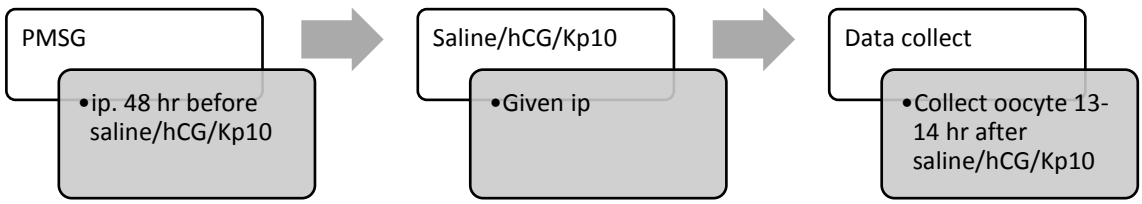
sacrificed 13-14 hour after the saline treatment by cervical dislocation, oviducts were removed and ova were recovered.

Kisspeptin protocol IV~VI. Mice received PMSG (eCG, 5 IU) by i.p. injection at day 1 and given three different levels (1 μg/100μl, 10 μg/100μl and 100 μg/100μl) of mkp-10 by i.p. injection 48 hours later (day3). The mice were sacrificed 13-14 hour after the mkp-10 treatment by cervical dislocation, oviducts were removed and ova were recovered.

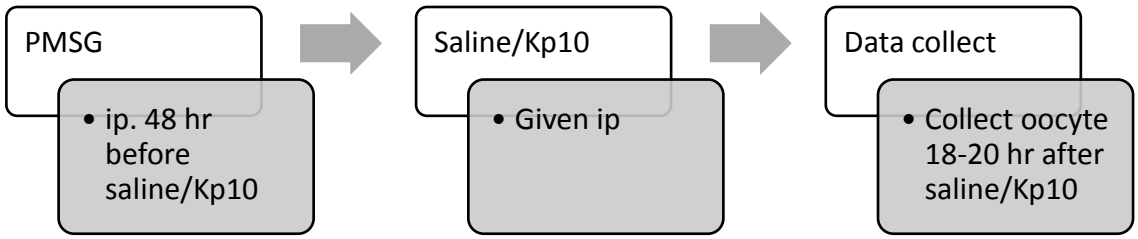
Conventional protocol. Mice received PMSG (eCG, 5 IU) by i.p. injection at day 1 and given hCG (10 IU) by i.p. injection 48 hours later (day3). The mice were sacrificed 13-14 hour after the hCG treatment by cervical dislocation, oviducts were removed and ova were recovered.



Prolonged retrieval time protocol. This is an additional protocol for further investigation about the effect of single i.p. kisspeptin to ovaries upon time. Mice received PMSG (eCG, 5 IU) by i.p. injection at day 1 and given saline 100μl or

doi:10.6342/NTU201703852

kisspeptin-10 100 µg/100µl 48 hours later (day3). The mice were sacrificed 18-20 hour later after the saline or kisspeptin injection by cervical dislocation, oviducts were removed and ovulated oocytes were recovered.



3.5 Tissue collection and histology assessment

Left and right ovaries were collected and fixed in 10% paraformaldehyde (PFA) overnight and then processed through graded alcohols into paraffin wax. Paraffin-embedded tissue sections were sectioned at 5 µm and stained with H&E stain.

3.6 Statistical analyses

Data are presented as the mean ± SD. The total numbers of oocytes collected from each mouse were evaluated statistically by two-way analysis of variance (ANOVA) using Sigma Plot software. The comparisons between groups were analysis by Tukey test. The rates of ovulation showed as percentage. Other statistical analyses are described as appropriate in the text or table footnotes. $P < 0.05$ was considered statistically significant.

doi:10.6342/NTU201703852

4. Results

To examine the effect of exogenous gonadotropin and kisspeptin-10 on mice, we compared the retrieved oocyte number using mice superovulation protocol and the altered protocols. The morphology of the ovaries between different treatment groups were observed under microscope. If the animal has a successful superovulatory stimulation or a normal ovulation in the control group, the ampulla of the oviduct will be larger and semi-transparent under the microscope (Figure 3A). The enlarge ampulla is stuffed with cumulus-oocyte-mass (COM). We use a 28G needle to tear the ampulla open and drag the COM in to a drop of 0.1% hyaluronidase in PBS buffer (Figure 3B). Incubate the drop for few minutes at room temperature and the cumulus cell will be digest by the enzyme and the oocyte is revealed (Figure 3C).

Since we pick the mice randomly and we didn't synchronize the estrus cycle before the treatments, corpus luteum from the last estrus cycle could be seen occasionally (Figure 3D). Some abnormal ovary morphology could happen when the animal was treated with PMSG, both in PMSG substituted trial and hCG substituted trial. For instance, the hemorrhagic follicle near the surface of ovaries (Figure 3E) or inside the

doi:10.6342/NTU201703852

ovary; the fluid-filled ovary bursa can also be detected (Figure 3F). The fluid is sometimes transparent or as red as blood.

4.1 The Effect of Long-term Subcutaneously Release of Kisspeptin-10 in Substituting for PMSG.

We use micro-osmotic pump to sustain the effect of kisspeptin-10 compared to PMSG in animals. Female ICR mice at 9-12 weeks of age were used. In control group, 50% (3/6) were ovulated; and three different kisspeptin release dose groups were all 83% (5/6) ovulated, while the PMSG positive control group was 100% (6/6) examined with ovulation (Figure 4A). The total collected oocyte numbers were 37, 63, 66, 71, and 172, respectively (Table 1). The average collected oocyte numbers were 6.17±7.0 for saline (n = 6), 10.5±4.8 for kisspeptin low dose (n = 6), 11.0±5.5 for kisspeptin medium dose (n = 6), 11.8±6.5 for kisspeptin high dose (n = 6), and 28.7±17.0 for PMSG positive control group (n = 6) (Figure 4B). The PMSG positive control group has more oocytes retrieved compared to other four treatments which is statistically significant ($p<0.05$). Since mice are ploy-ovulated animal, a successful pregnancy would need several oocytes to stimulate a maternal recognition. If the retrieved oocyte number was greater than 7, then the animal was recognized as a successful ovulated individual. The

doi:10.6342/NTU201703852

average retrieved oocyte per successfully ovulated mouse were 12.0±4.4 for saline control group (n = 3), 11.8±4.1 for kisspeptin 1 μg protocol (n = 5), 13.2±1.3 for kissepetin 10 μg protocol (n = 5), 14±4.2 for kisspeptin 100 μg protocol (n = 5), and 28.7±17.0 for PMSG positive control protocol (n = 6). Only kisspeptin 1 μg treatment statiscally yields less oocytes compared to PMSG positive control group ($p<0.05$). And all kisspeptin treatment groups are not statistically different compared to saline control group (Figure 4C).

To examine the effects of long-term stimulation of kisspeptin-10 on the histological morphologies of female ovaries in mice, the mice ovaries were collected at the time with the oocyte number counted. As shown by the results of a histological analysis by hematoxylin and eosin (H&E) staining (Figure 7), morphological changes in the ovaries were induced by different treatments. The saline-hCG treatment group had various ovary morphologies depend on the animals' nature estrus cycle. Some ovaries were with more developing follicles (Figure 7A), while others have more corpus luteum (Figure 7B). The kisspeptin-10-hCG treatments have both corpus luteum and developing antral follicles (Figure 7C, 7D, 7E), some with Graafian follicles (Figure 7C). The corpus luteum and antral follicles share the average space in the ovary. The

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number of corpus luteum was increased by PMSG-hCG treatment compare to other protocols (Figure 7F). The corpus luteum occupied most of the space in the ovaries, with very few developing antral follicles.

4.2 The Effect of Using Single Intraperitoneal Injection of Kisspeptin-10 to replace hCG.

In the second part of the experiment we use an intraperitoneal injection of kisspeptin-10 to mimic the administration of hCG in superovulation protocol. Female mice at 9-12 weeks of age were used. In PMSG-saline control group, about 20% (2/10) were ovulated, the kisspeptin-10 1 μg treatment group had 25% (1/4) of ovulation, while the other two kisspeptin higher dose treatment (10 μg and 100 μg) had no oocyte retrieved (0% ovulated, 0/4 and 0/8 respectively). On the other hand, all the PMSG-hCG treated mice were found ovulated (100%, 8/8) (Figure 5A). The total collected oocytes were 44, 16, 0, 0 and 254, respectively (Table 2). The average collected oocyte number were 4.4±10.6 in PMSG-saline group (n = 10), 4.0±8.0 in PMSG-kisspeptin-10 1 μg treatment (n = 4), 31.8±18.3 in PMSG-hCG positive control group (n = 8), and no oocyte was recovered in other two kisspeptin-10 higher dose treatments (Figure 5B). Only the PMSG-hCG positive control group significantly yields

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more oocytes compare to other group ($p<0.05$). Since the number of successfully ovulated animal is too small between groups (n = 2; n = 1; n = 8), no statistical analysis was performed.

To confirm the effect of kisspeptin given single dose with an i.p injection to the ovaries, we did another trial with a longer interval between kisspeptin injection and oocyte retrieval. The original time period 13-14 hour after the treatment was prolonged to 18-20 hour. The saline control group and kisspeptin-10 group had the same ovulation rate at 33% (2/6) (Figure 6A), while the average oocyte retrieval number were 7.5 ± 11.6 and 4 ± 6.5 (Figure 6B). There was no significance difference between two groups.

To examine the effect of single i.p inject of kisspeptin-10 on the histological morphologies of female ovaries in mice, the ovaries were collected at the time when the oocyte number were counted. As shown by the results of a histological analysis by hematoxylin and eosin (H&E) staining (Figure 8), morphological changes in the ovaries were induced by different treatments. In the PMSG-saline treatment group, the ovaries we collected showed more corpus luteum than mature follicles. Most ovaries area was occupied by large corpus luteum, with some developing follicles (Figure 8A). The morphology of PMSG-kisspeptin-10 1 μg group shows newly formed corpus luteum

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and many early antral follicles (Figure 8B). The PMSG-kisspeptin-10 10 μg group had more corpus luteum and antral follicle compared to other groups while the sizes are smaller (Figure 8C). The PMSG-kisspeptin-100 1 μg group have large antral follicle and corpus luteum (Figure 8D). The PMSG-hCG positive control group had most of the ovary area occupied by corpus luteum (Figure 8E).

Occasionally we found some polyovular follicle in these ovaries (Figure 9A). It is considered a normal case in young mice. While the hemorrhagic follicles exist in every PMSG treated groups, including PMSG-saline treatment (Figure 9B), PMSG-kisspeptin-10 1 μg group (Figure 9C), PMSG-kisspeptin-10 10 μg group (Figure 9D), PMSG-kisspeptin-10 100 μg group (Figure 9E), and PMSG-hCG conventional protocol (Figure 9F). While no kisspeptin-10-hCG treated animals in PMSG substituted trial found hemorrhagic follicle.

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5. Discussion

The conventional ovary-stimulated protocol is well established and mainly rely on the use of exogenous gonadotropins. PMSG and hCG are two most popular exogenous gonadotropins owing to the long half-life make it more efficient and convenient in ART. However, the use of PMSG and hCG over stimulate the ovary and cause damage to female reproduction tract. Moreover, the development of antibodies of PMSG and hCG are found in animals after multiple treatments, which cause the treatment efficiency drop. These phenomena imply that the protocol is not sustainable for livestock reproduction management. On the other hand, kisspeptin is reported to have gonadotropic activity both given centrally and peripherally. The aim of this study is to access the feasibility of using kisspeptin to substitute for PMSG or hCG.

5.1 The Effect of Long-term Subcutaneously Release of Kisspeptin-10 in Substituting for PMSG.

The mice superovulation protocol was altered to assess the effect of kisspeptin on female mice ovary. We use osmotic-mini pump to treat the animal subcutaneously to overcome the short half-life of kisspeptin. Former research mainly focuses on the central response via the icv administration of kisspeptin or given intravenously. The

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elevated LH and FSH were reported when animals were given kisspeptin centrally or peripherally. Although we didn't determine the serum LH and FSH levels in our experiment, the secretion of LH and FSH is expected to be affected through the exogenous kisspeptin. Since there are more animals ovulated in the kisspeptin long-term treated group compared to the control one.

In the first part of the study, we demonstrated the long-term release of kisspeptin via osmotic-mini pump could stimulate the development of follicle. The kisspeptin treated group have more ovulated individuals compared to saline treated control group. This result implies that kisspeptin could be used on animal estrus cycle synchronization. The recovered oocyte number, although is not as much as the conventional PMSG-hCG protocol, is as normal as the saline control group, and the variation is smaller compared to the conventional protocol. This merit implies the feasibility of kisspeptin substitute for PMSG, since one of the drawback of PMSG is the high variation among individuals. Moreover, there are no cases of over-stimulated ovary or hemorrhagic follicle found in long-term kisspeptin stimulated treatment, while both cases happen in PMSG treated animal. In PMSG substitution trial, we found that the subcutaneously administration of

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kisspeptin can synchronize the estrus cycle and mildly stimulate the ovary and promote follicle development.

Occasionally we found some polyovular follicle in kisspeptin treated group. Individual follicles may be closely adjacent to each other. Each of these follicles contains one oocyte. A polyovular follicle comprises a single follicle containing two oocytes, each with its own nucleus, zona pellucida, and layer of granulosa cells. Polyovular follicles are occasionally seen as a background finding. Since the number of polyovular follicle is not much, it is considered a normal case in estrous cycle.

Swollen ovary and hemorrhagic follicle are the phenotype of ovarian hyperstimulation syndrome (OHSS) and polycystic ovarian syndrome (PCOS). The cases of hemorrhagic follicle are reported in estrogen receptor-α (Esr1) knockout mice. Esr1 is mainly expressed in the theca/interstitial cells and germinal epithelium and thus is believed to mediate estrogen action in these cells. Esr1KO mice display severe ovarian disorders such as disrupted theca/stromal development, arrest of follicular development at early antral stage, and formation of hemorrhagic cysts (Lee *et al.,* 2009). Young PKBβ (also known as Akt2) knockout mice were used to model PCOS by treatment with LH and exhibited a cyst area that was threefold greater than in controls

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(Restuccia *et al.*, 2012). Overexpression of FSHβ leads to detectable numbers of cystic or hemorrhagic follicles without changes in circulating LH levels. Treatment with letrozole, an aromatase cytochrome P450 (P450arom) blocker, inhibits androgen-to-estrogen conversion and leads to the development of massive multiple follicular cysts in rats. ERβ is required for the development of hemorrhagic and cystic follicles in the ovary during chronic LH stimulation (Couse *et al.*, 2004). The use of PMSG is also one of the method to induce OHSS. The hemorrhagic follicles we found in PMSG treated ovaries might because the long half-life of PMSG irritated the follicle development, and sometimes the stimulation is so severe and might be the reason why we found hemorrhagic ovaries from time to time.

5.2 The Effect of Using Single Intraperitoneal Injection of Kisspeptin-10 to replace hCG.

In the experiment of replacing hCG, we found that the single ip injection of kisspeptin somehow regulate ovulation and reduce oocyte yield compared to control group. Surprisingly, we didn't retrieve any oocyte in 10 μg and 100 μg kisspeptin protocol. The injection was not invalid because of the short half-life of kisspeptin, otherwise the ovulation rate should be similar to the saline control group. But we can’t

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be sure if the single bolus of kisspeptin ip injection can somehow block the ovulation or prolong the ovulation time. Therefore, another trial was conducted to confirm if kisspeptin block the ovulation or it just needed more time to induce oocyte maturation and promote ovulation. In the prolonged retrieval protocol, we prolonged the retrieval time from 13-14 hour to 18-20 hour. The results indicated that there were no differences between saline control group and kisspeptin treatment group.

The number of animal we used in kisspeptin 1 μg group and 10 μg group should be more to confirm the result statistically. In other studies, it was reported that only one single iv or icv injection of kisspeptin could lead to the elevation of LH and FSH levels. In our experiment, we didn't access the level of LH and FSH of mice during the experiment; therefore, it is uncertain whether the treatment promotes the LH and FSH level or suppresses the secretion of these gonadotropins through central mechanism. The central control of kisspeptin system has two mechanisms during the estrous cycle depending on the location of the kisspeptin neuron. The kisspeptin neurons at the AVPV area prone to stimulate the GnRH neurons during estrus and induce a preovulatory GnRH surge, while the kisspeptin neurons located at the ARC area stay at low level during LH surge and maintain GnRH at a basal pulsatile level. If the

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exogenous kisspeptin suppress the ovulation through central pathway, the route should be ARC-GnRH- low LH level which cannot induce ovulation. However, since the half-life of kisspeptin in vivo is relatively short, single ip injection of kisspeptin should be degraded before it could reach the central and regulate GnRH secretion.

Another possibility of suppressing the ovulation by the single inject of kisspeptin could be the locally peripheral inhibition. The expression of kisspeptin receptor not only locate at the central nerve system, but also in gonads. Although the exact location of the Kiss1R is still unknown, the role of kisspeptin system in ovary is controversial. Specific gonad Kiss1 KO mice will loss fertility, failed to puberty and the development of reproductive system is blocked, which indicate the importance of kisspeptin signal to maintain the normal reproductive function. On the other hand, a study in female rat found elevated kisspeptin expression in aged rats compared to the young ones (Fernandois *et al.*, 2016). The results show that kisspeptin expression in the ovary was increased in 10- and 12-month-old rats compared with 6-month-old rats, and this increase in kisspeptin was strongly correlated with the increase in ovarian norepinephrine observed with aging. The administration of kisspeptin produced an increase in Corpus lutea and type III follicles. In addition, kisspeptin decreased the

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number and size of antral follicles. These theories show that the regulation of local kisspeptin to ovary involves many elements, and the reason of our results still require further examination to confirm the effect of single injection of kisspeptin on the ovary and follicle development.

Most superovulation research will further examine the quality of oocyte, in our study we just count the oocyte number and examine the morphology under the microscope. Most of the oocytes look normal and in good quality. But still, further work such as parthenogenetic activation or IVF could be done to clarify the kisspeptin-induced oocytes are good in quality for embryology research or ART.

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6. Conclusion

Our study conducts the long-term stimulation of kisspeptin which given subcutaneously has the potential of substituting the use of PMSG. The long-term kisspeptin stimulation provides a milder impact on the ovary and smaller variation compared to PMSG. The next goal for the substitution of PMSG is to overcome the short half-life of kisspeptin-10. Modify the peptide to prolong the half-life or designed a time-released dosage form are two main strategies. For the hCG replacement trial, the ovulation is prolonged by the administration of kisspeptin. The mechanism underlying this result remains unknown and require further investigation.

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7. Figures and Tables

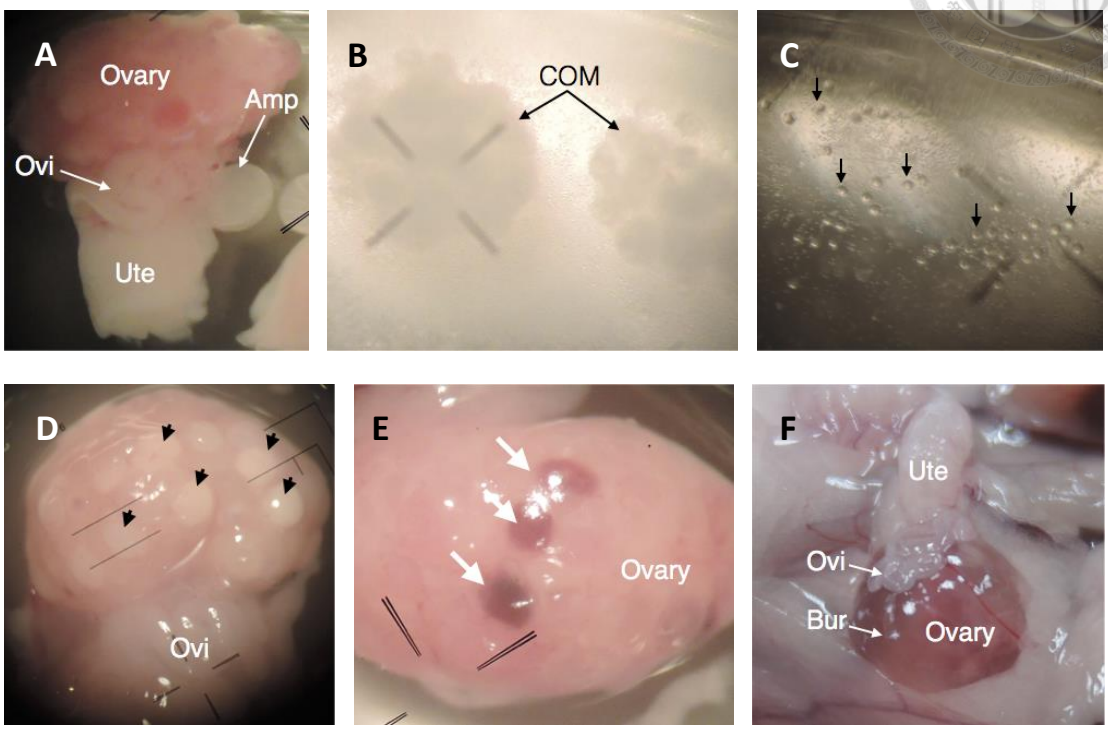

Figure 3. (A) The morphology of mouse ovaries and oviducts with visible ampulla. (B) The cumulus oocyte mass (COM) in the ampulla. (C) The oocytes (black arrows) shows after the COM treated with 0.1% hyaluronidase. (D) A mouse ovary with corpus luteum (black arrowhead). (E) The PMSG treated ovary with hemorrhagic follicles (while arrows). (F) The PMSG treated ovary in the fluid-filled swollen bursa membrane. Amp, ampulla; Bur, bursa membrane; Ovi, oviduct; Ute, uterine horn.

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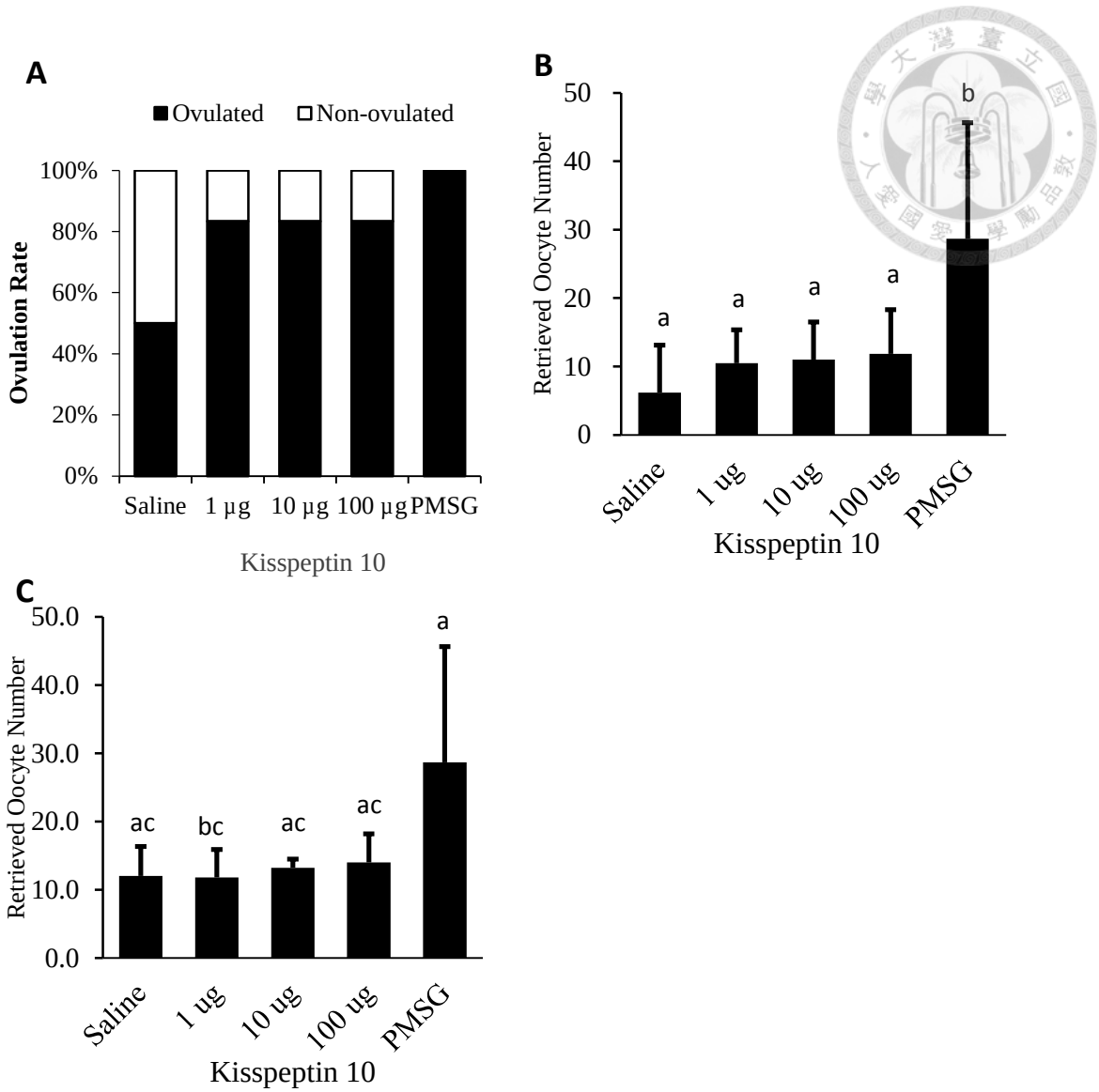

Figure 4. (A) The ovulated rate between different treatment in PMSG substitute trial. Saline (50%), Kisspeptin-10 1 µg (83%), Kisspeptin-10 10 µg (83%), Kisspeptin-10 100 µg (83%) and PMSG (100%). (n=6). (B) Average retrieved oocyte number in PMSG substitute trial (n=6). (C) Average retrieved oocyte number in successfully super ovulated individual (n=3~6).

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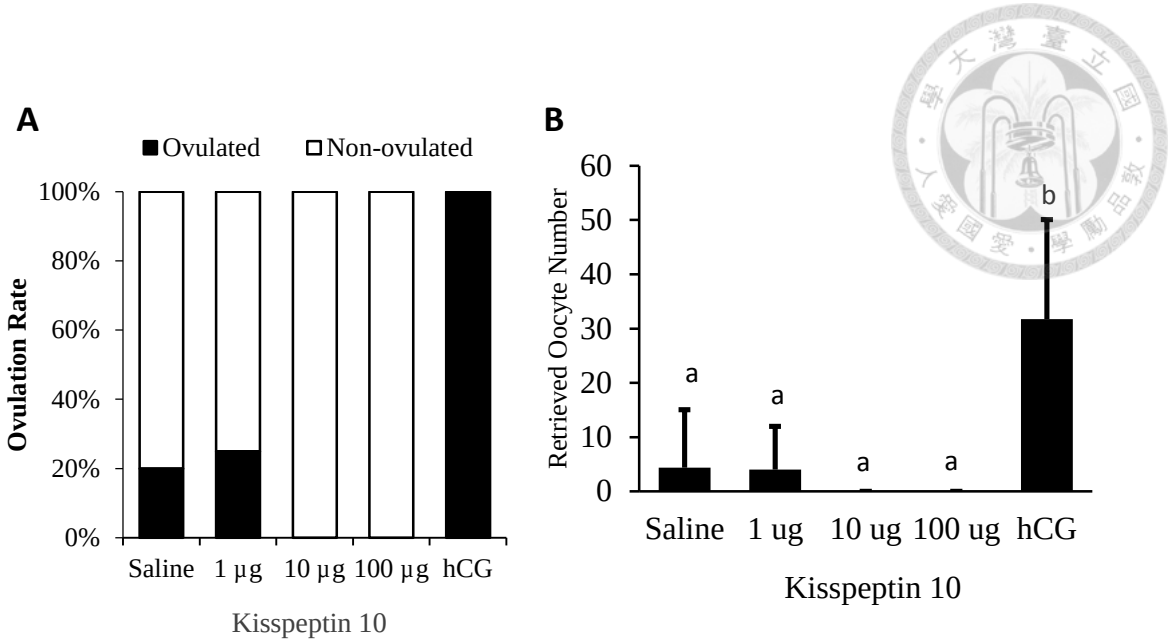

Figure 5. (A) The ovulated rate between different treatment in hCG substitute trial. Saline (20%), Kisspeptin-10 1 μg (25%), Kisspeptin-10 10 μg (0%), Kisspeptin-10 100 μg (0%) and hCG (100%). (n=4~10) (B) Average retrieved oocyte number in hCG substitute trial (n=4~10). Data show as mean ± SD.

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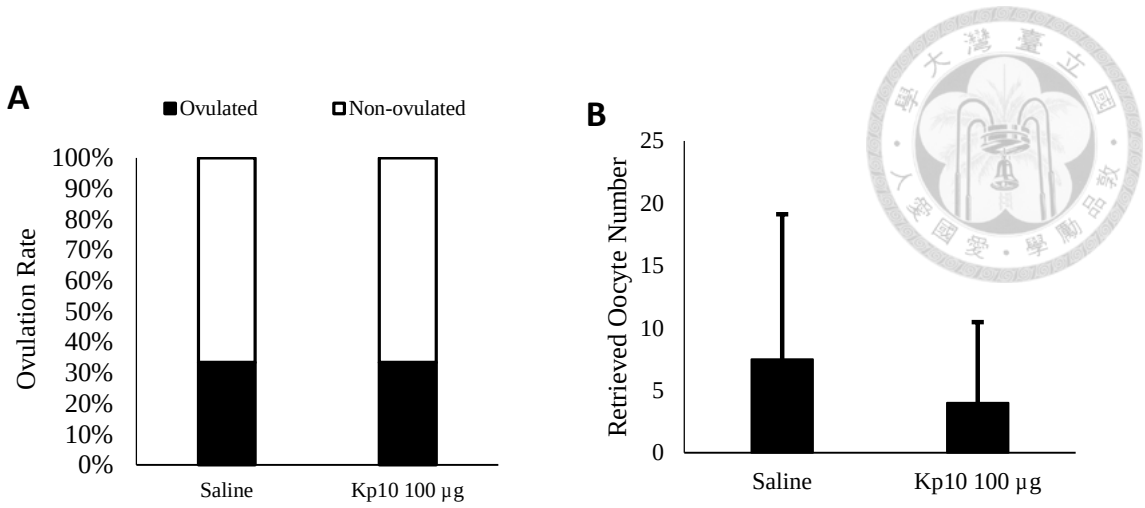

Figure 6. (A) The ovulated rate between different treatment in prolonged retrieval time trial. Saline (33%) and Kisspeptin-10 100 µg (33%) (n=6). (B) Average retrieved oocyte number in prolonged retrieval time trial (n=6). For saline group 7.5 ± 11.6 and Kp-10 group 4 ± 6.5. Data show as mean ± SD.

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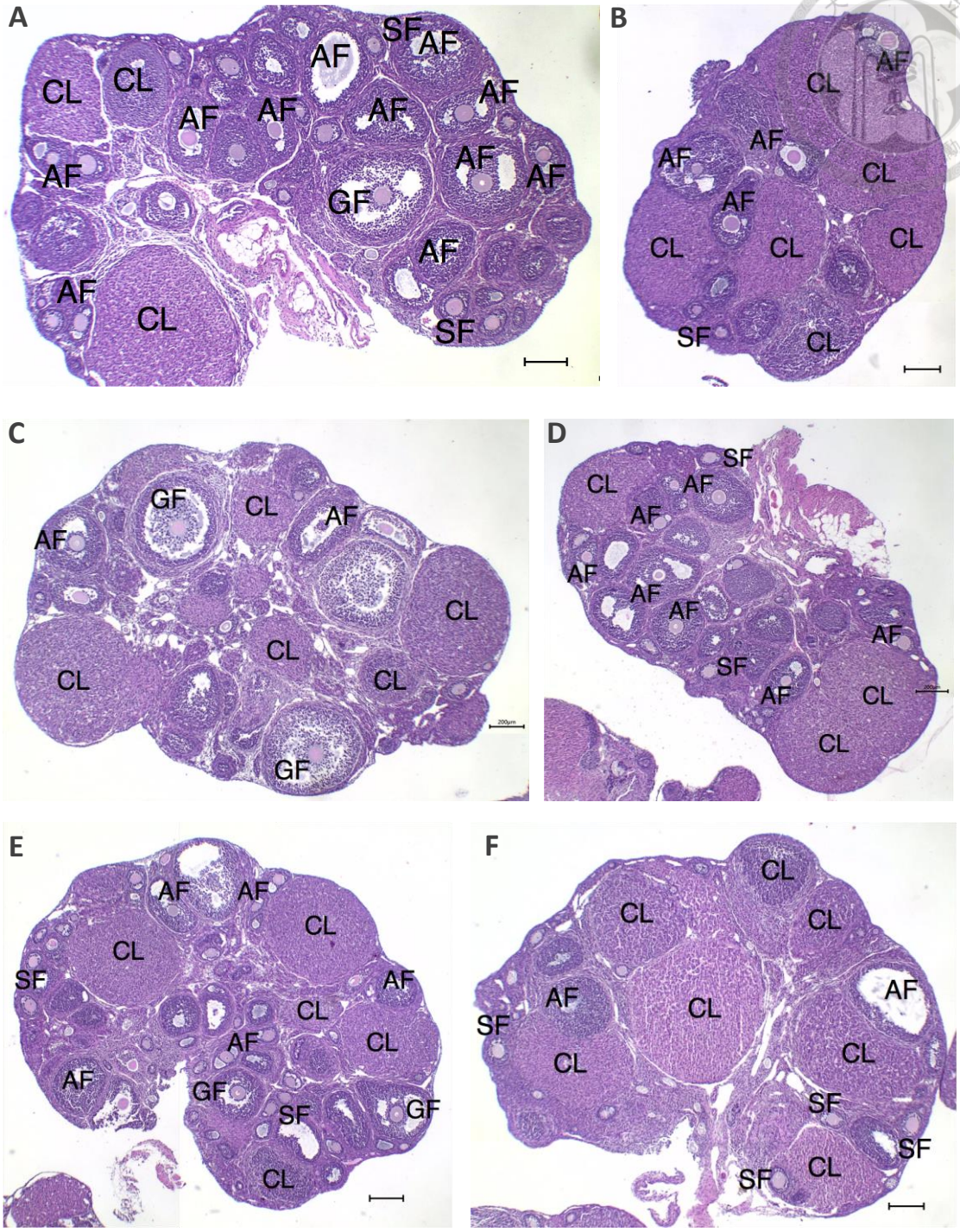

Figure 7. The representative histopathological pictures of ovaries stained by H&E in PMSG substitute trial. (A) and (B) Saline-hCG. (C) Kisspeptin-10 1 μg-hCG. (D) Kisspeptin-10 10 μg-hCG. (E) Kisspeptin-10 100 μg-hCG. (F) PMSG-hCG. Bar = 200 μm. PF, primary follicle; SF, secondary follicle; AF, antral follicle; GF, Graafian follicle; CL, corpus luteum.

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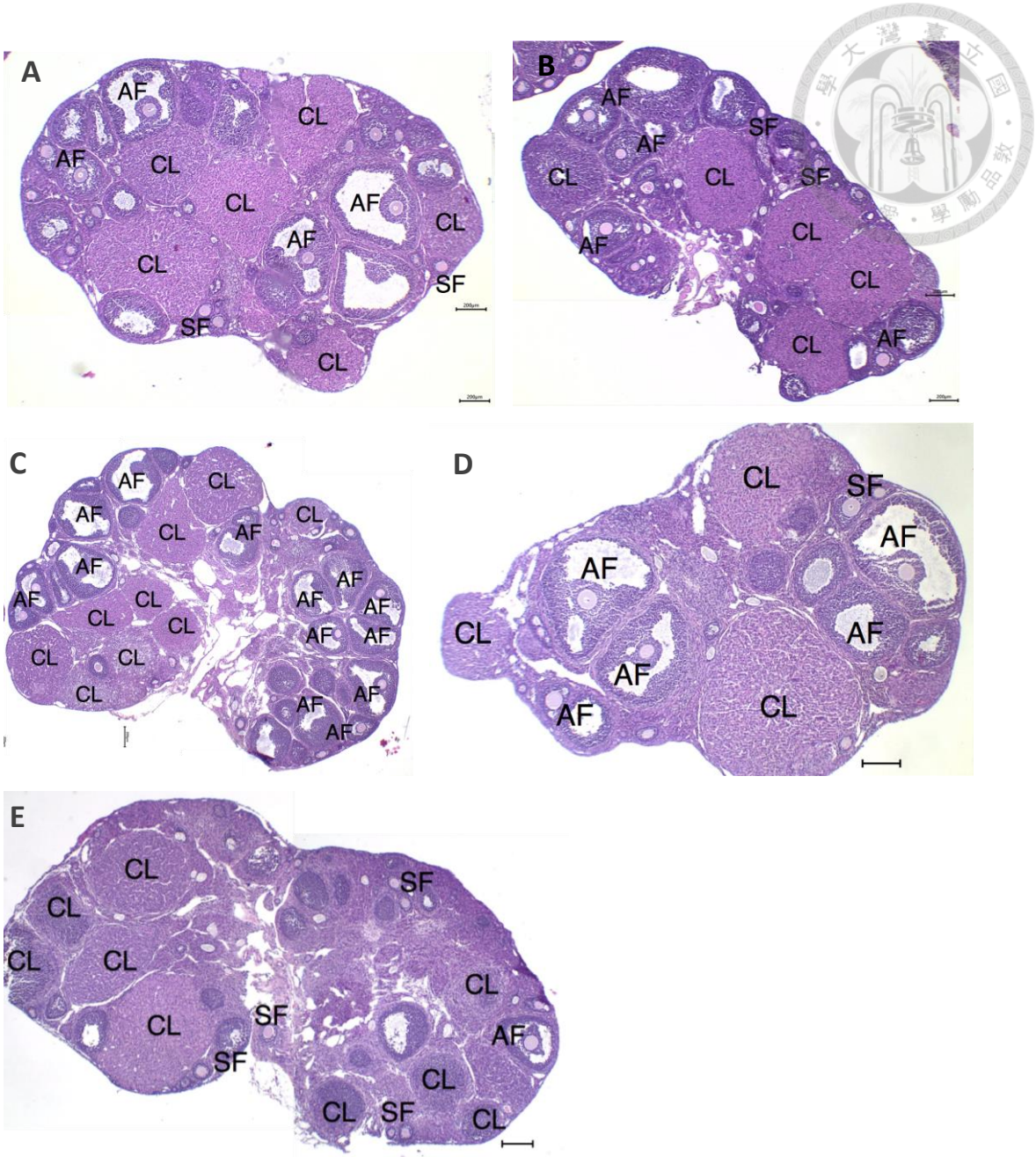

Figure 8. The representative histopathological pictures of ovaries stained by H&E in hCG substitute trial. (A) PMSG-saline. (B) PMSG-Kisspeptin-10 1 μg. (C) PMSG-Kisspeptin-10 10 μg. (D) PMSG-Kisspeptin-10 100 μg. (E) PMSG-hCG. Bar = 200 μm. PF, primary follicle; SF, secondary follicle; AF, antral follicle; GF, Graafian follicle; CL, corpus luteum.

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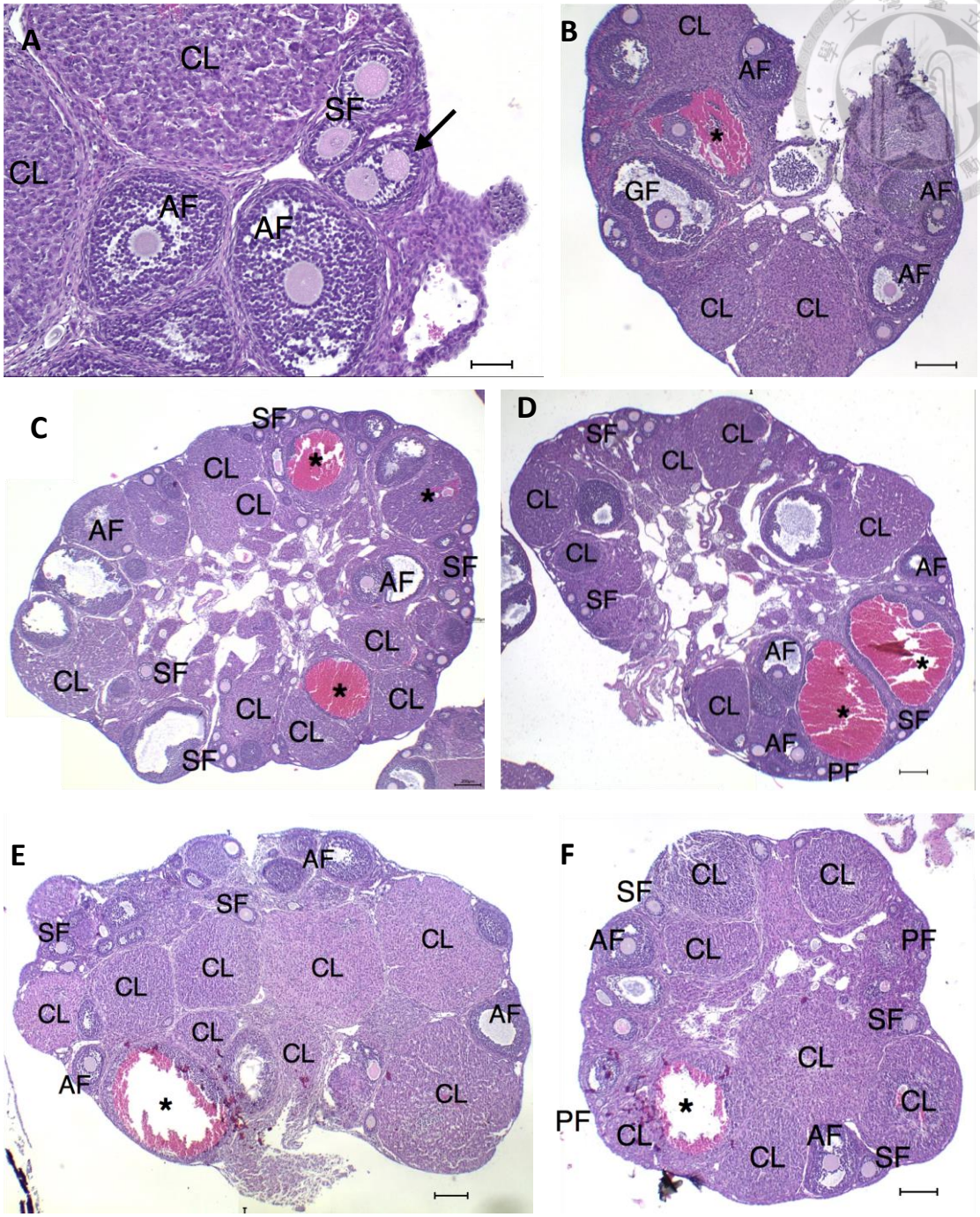

Figure 9. Normal and abnormal follicle. (A) Polyovular follicle (black arrow). Bar = 100 µm. (B) PMSG-saline treatment ovary. (C) PMSG-Kisspeptin-10 1 µg treatment. (D) PMSG-Kisspeptin-10 10 µg treatment. (E) PMSG-Kisspeptin-10 100 µg treatment. (F) PMSG-hCG treatment. PF, primary follicle; SF, secondary follicle; AF, antral follicle; GF, Graafian follicle; CL, corpus luteum. Asterisks denote hemorrhagic follicles. Bar = 200 µm.

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Table 1. The ovulation ratio and oocyte number among different treatments in PMSG substitute trial.

Treatment Group	Total No. of retrieved oocyte	Average oocyte No.	Ovulation Ratio (ovulated /total) (retrieved oocyte No. >6)	Ovulated mice	
				Total No. of oocyte	Average oocyte No.
Saline	37	6.17±7.0 [a]	50% (3/6)	36	12.0±4.4 [a,c]
Kp-10 1 μg	63	10.5±4.8 [a]	83% (5/6)	59	11.8±4.1 [b,c]
Kp-10 10 μg	66	11.0±5.5 [a]	83% (5/6)	66	13.2±1.3 [a,c]
Kp-10 100 μg	71	11.8±6.5 [a]	83% (5/6)	70	14±4.2 [a,c]
PMSG	172	28.7±17.0 [b]	100% (6/6)	172	28.7±17.0 [a]

Oocyte number are expressed as means ± SD (n=3~6). Multiple Comparison (Tukey Method) [a,b,c] Values with different superscripts within the same column are different ($P<0.05$).

Table 2. The ovulation ratio and oocyte number among different treatments in hCG substitute trial.

Treatment Group	Total No. of retrieved oocyte	Average oocyte No.	Ovulation Ratio (ovulated /total) (retrieved oocyte No. >6)	Ovulated mice	
				Total No. of oocyte	Average oocyte No.
Saline	44	4.4±10.6 [a]	20% (2/10)	44	22.0±15.6
Kp-10 1 μg	16	4.0±8.0 [a]	25% (1/4)	16	16
Kp-10 10 μg	0	0.0±0.0 [a]	0% (0/4)	0	0
Kp-10 100 μg	0	0.0±0.0 [a]	0% (0/8)	0	0
hCG	254	31.8±18.3 [b]	100% (8/8)	254	31.8±18.3

Oocyte number are expressed as means ± SD (n=4~10). Multiple Comparison (Tukey Method) [a,b] Values with different superscripts within the same column are different ($P<0.05$).

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