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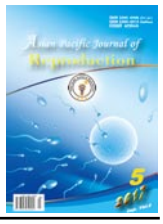

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Effects of anestrus dog serum on superovulation in rats and mice

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ABSTRACT

Objective: To study the effects of anestrus dog serum in rodents (contains high amount of FSH) compared with two common superovulatory programs. **Methods:** Rats (n=30) at diestrus phase were evenly divided into pregnant mare serum gonadotrophins (PMSG) group (administrated with 30 IU PMSG, and 48 h later following by 25 IU hCG), recombinant follicle stimulating hormone (rFSH) group (reducing dose every 12 h from 5 to 1 IU, then following by 25 IU hCG) and anestrus dog serum group (reducing dose every 12 h from 0.6 to 0.1 mL, then following by 25 IU of hCG). Mice (n=30) were also evenly divided into PMSG group (5 IU PMSG used at 13 pm, and 48 h later following by 5 IU of hCG), rFSH group (from 13 pm, reducing dose every 12 h from 2.5 to 0.5 IU, then following by 5 IU hCG) and anestrous dog serum group (from 13 pm, reducing dose every 12 h from 0.100 to 0.025 mL, then following by 5 IU hCG). Mice and rats placed with males for 24 h after last injection. Histology samples of ovaries were prepared and the numbers of corpus lutea were counted on day 14 after mating. **Results:** In mice, the differences among mean number of corpus lutea in all groups (P=0.01) and between FSH and dog serum group were significant (P=0.000 7). But no significant differences were found between the mean number of corpus lutea in dog serum and PMSG, or between FSH and PMSG groups. In rats, mean number of corpus lutea were significant differences among three groups (P=0.01), and between PMSG and dog serum groups (P=0.02). **Conclusions:** Superovulatory response in anestrus dog serum group is similar to PMSG group in mice, which is relatively similar to FSH in rats.

1. Introduction

Superovulation has been used for the production of the large number of embryos to maximize the reproductive efficiency in different species of animals[1]. In farm animals, superovulation and embryo transfer are routinely used as an efficient commercial assisted reproductive technology. Likewise, in laboratory animals, induction of superovulation results in ovulation of large numbers of oocytes from limited numbers of females which in turn facilitates the generation of genetically engineered animals[2-4]. Improving superovulatory response with the least variation in the production of embryos has been an important objective in the field of research in reproductive technology in different species of farm as well as laboratory animals. Pregnant mare serum gonadotrophins (PMSG) and follicle stimulating hormone (FSH) are two commercially used hormone products that are routinely used for induction of superovulation in rats and mice[5]. However, the significant variation in the response to different superovulatory protocols is a serious challenge. Many researches showed that the variation in

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the superovulatory response may result from genetic differences among strains, type of the hormone, schedule for the hormone administration and dosage of the hormone[6].

PMSG products have an approximately 5 d half-life in blood circulation. This causes emergence of the new follicular waves after ovulation and then high estrogen production[7]. The residue of PMSG may have a continuous superovulatory effect and this has negative effect on the rates of ovulation, fertilization and embryo development[8]. In contrast to PMSG, the half-life of FSH preparations in the blood circulation is approximately 3-4 h[6]. Therefore, the superovulatory response using FSH preparations are more consistent and predictable than PMSG preparations. There are various types of FSH preparations such as human menopausal gonadotrophins, purified pituitary extract of porcine FSH, and ovine FSH[9]. With the advent of recombinant DNA technology, a new horizon emerged for preparation and production of recombinant FSH (rFSH) by culturing the ovary cells of Chinese hamster[10]. Now, rFSH is frequently used for superovulatory treatments. It is also important to mention that the cost of rFSH is higher than other FSH preparations.

A large of amount of FSH is present in the blood circulation of anestrous dogs. This is due to the ovarian inactivity and lack of negative feedback effects of estrogen and progesterone on the hypothalamus and pituitary. Anestrous is a stage occurs between the end of diestrus or whelping and the beginning of next proestrous. This period lasts 4-5 mo in average or can extend up to 1 year[11,12]. The mean serum FSH concentration in anestrous stage was reported in the range of (240±18) ng/mL up to (294±52) ng/mL[13,14]. To our knowledge, there is no study examining the effect of anestrous dog's serum for induction of superovulation. Therefore, the present study was designed to compare the superovulatory response following treatment with unpurified dog's anestrous serum, PMSG and rFSH in adult BALB/c mice and Sprague Dawley rats.

2. Materials and methods

This study has been approved by the Iranian laboratory animal ethics framework under the supervision of the Iranian Society for the Prevention of Cruelty to Animals and Shiraz University Research Council.

2.1. Serum preparation of anestrous dog

Three healthy adult intact female dogs were selected. Appropriate, standard food and antiparasitic drugs were given for at least 2 wk before study. Their anestrous status was confirmed by vaginal cytology and concentration of progesterone (P4<1 ng/mL)[11]. Then, maximum available blood was calculated (10%) based on their body weights. The blood collected with placing catheter in jugular vein. The blood reserved in separate collecting tubes (10 mL). Immediately, the serum of the blood was centrifuge 3 000 r/min for 10 min and stored at -20 ℃. The volume of the serum was about 30% of the blood volume.

The laboratory animals were prepared from the Laboratory Animal Center of Shiraz University of Medical Sciences. As a routine program, the colony is strictly maintained by inbreeding. Males and females were separated before puberty at the age of 21 d. They were kept at special cages (27 cm×21 cm×14 cm) and suitable rooms (22 ℃, 40%-60% humidity, 12 h of light and 12 h of darkness) and fed with standard pelleted ration (laboratory animal feed, Javaneh Khorasan, Iran). In this study, female BALB/c mice aged 6-8 wk and fertile male mice aged 2-3 mo were selected and (Experiment Ⅰ). Male and female rats of Sprague Dawley species aged 10-12 wk were selected (Experiment Ⅱ).

2.2. Superovulation protocols

Experiment Ⅰ: Female mice at diestrus stage were divided into three groups: Group 1 (n=10) were administered with 5 IU PMSG (Pregnecol, Bioniche Animal Health, Australia) followed by 5 IU hCG (DarouPakhsh, Iran) 48 h later. Group 2 (n=10) were administered with 7.5 IU rFSH (Fostimon, IBSA, Switzerland) in decreasing dose regimen (2.5, 2.0, 1.5, 1.0 and 0.5 IU) in a 48 h period every 12-hour followed by 5 IU of hCG. Group 3 (n=10) were administered with 0.28 mL anestrous dog serum in decreasing dose regimen (0.100, 0.075, 0.050, 0.030 and 0.025 mL) in a 48 h period every 12-hour followed by 5 IU hCG. The amount of anestrus dog's serum was considered as volume equivalent to rFSH dose in group 2 (2.5 IU rFSH = 0.1 mL dog serum).

Experiment Ⅱ: Rats at diestrus stage were also divided into three groups: Group 1 (n=10) received 30 IU PMSG, and 48 h later following by 25 IU hCG. Group 2 (n=10) received 15 IU rFSH in decreasing dose regimen (5, 4, 3, 2 and 1 IU), and 48 h later following by 25 IU hCG every 12 h in a 48 h period. Group 3 (n=10) received 1.6 mL anoestrous dog serum in decreasing dose regimen (0.6, 0.4, 0.3, 0.2 and 0.1 mL), and 48 h later following by 25 IU hCG every 12 h in a 48 h period. All hormone and serum administrations were performed intraperitoeously. All of animals were mated with a ration 1:1 (female:male) after hCG administration for 24 h. It should be noted again that the volume of anestrus dog's serum injection calculated based on equivalent volume of rFSH in group 2 (5 IU rFSH =0.6 mL dog serum).

On day 14 after mating, animals were killed by cervical dislocation[15]. Sections of the ovary of mice and rats were fixed in buffered formalin 10% for histopathological evaluation and corpora lutea counting. Afterwards, slides were stained using Hematoxylin-Eosin method. The data were recorded for each animal and number of corpora lutea reported as mean±SEM. Because the pregnancy results were not the goal of this study, we only reported the number of gestational sac and corpora lutea in pregnant mice and rats without statistical analysis.

2.3. Statistical analysis

The results of two separate studies were analyzed with Graphpad Prism 6. As obtained data was quantitative and independent, they were grouped in three categories and one way ANOVA performed

to compare the means of corpora lutea in three groups and Tukey's multiple comparisons to compare group by group. The data were expressed as mean ± SEM and significant difference was considered at $P<0.05$.

3. Results

3.1. Experiment Ⅰ

The mean number of corpora lutea was 28.8 ± 5.18 in FSH group, 12.70 ± 1.52 in dog serum group and 23.60 ± 3.97 in PMSG group. Pregnancy was only observed in PMSG group and the numbers of embryos and corpora lutea in three mice of this group were 9 and 10, 15 and 24, 30, 48, respectively. In statistical analysis, the difference between the mean number of corpora lutea in all three groups was significant (P=0.011 7, Figure 1). Also, difference among the number of corpora lutea between FSH and dog serum group was significant (P=0.000 7), but no difference was found between dog serum and PMSG group or between FSH and PMSG group (Figure 2).

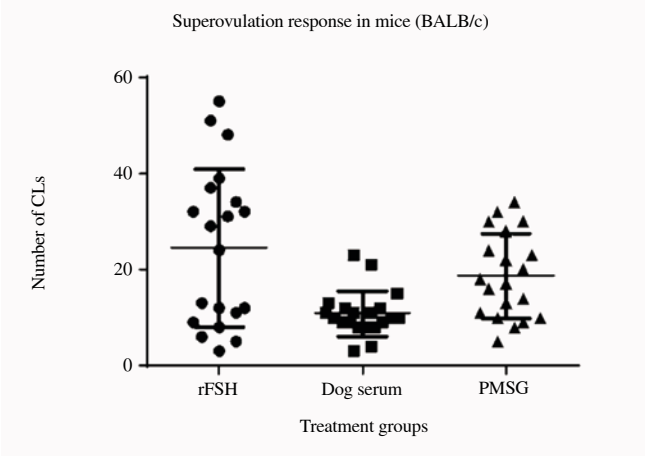

Figure 1. Mean numbers of corpora lutea 14 d after mating in PMSG, FSH and dog serum groups.

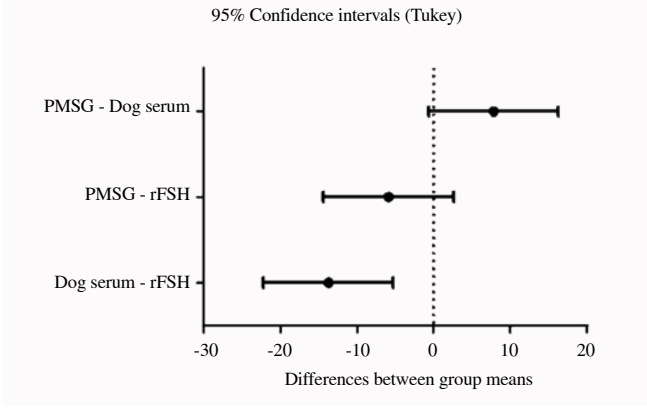

Figure 2. Differences in mean numbers of corpora lutea among mice treatment groups.

3.2. Experiment Ⅱ

In rats, comparing the number of corpora lutea showed significant difference among groups (P=0.01, Figure 3). Difference in the mean number of corpora lutea between PMSG group (35.10 ± 5.26) and dog serum (20.60 ± 2.57) was significant (P=0.02). There was no significant difference in corpora lutea numbers between FSH group (24.20 ± 2.00) and PMSG group (P=0.097), or between FSH and dog serum group (P=0.75, Figure 4). There was not pregnancy in rats of PMSG group. The number of embryos and corpora lutea in two rats of FSH group was 12, 26 and 18, 32 and in five rats of dog serum group were 5, 13, 7, 12, 7 and 19, 12, 15, 12, 24, respectively.

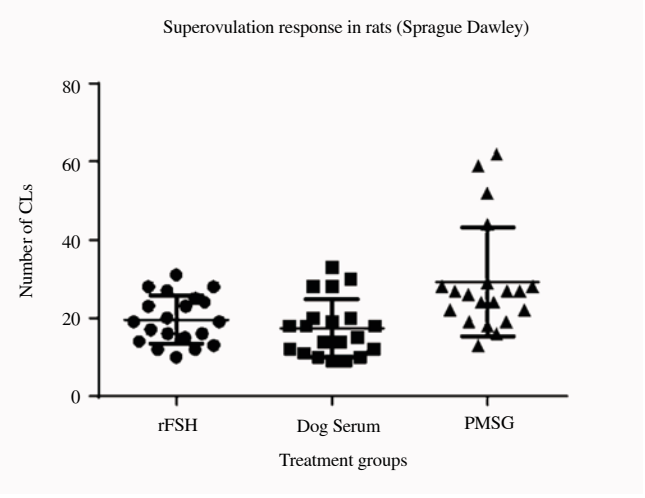

Figure 3. Mean numbers of corpora lutea 14 d after mating in PMSG, FSH and dog serum groups.

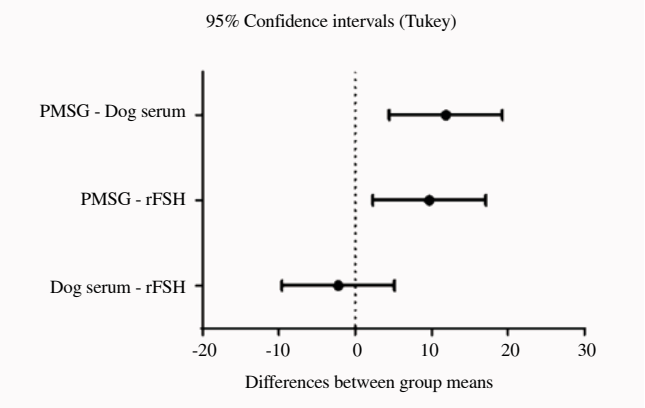

Figure 4. Differences in mean numbers of corpora lutea among rats treatment groups.

4. Discussion

In this study, the effects of anestrous dog's serum contains FSH hormone were compared with two conventional superovulatory

programs in rats and mice. The results showed that the response of anestrous dog serum in BALB/c mouse was similar to PMSG, but in rats the response was close to FSH group.

In this study, it was tried to prepare the best conditions for superovulation induction according to review literatures. The most appropriate dosage for induction of superovulation was suggested 5-10 IU of PMSG and hCG in BALB/c mice and 30 IU of PMSG and 5-10 IU of hCG in rats[16]. For FSH, maximum responses obtained at 2.5 to 0.5 IU with decreasing doses[17]. Suitable optimal interval time between two injections of PMSG and hCG hormones was reported to be 40-56 h[18]. Apart from the type of hormones, the best time of induction of superovulation in rats is at diestrus phase[17]. In mice, superovulation can be performed at all stages of estrus cycle. However, researches showed that estrus phase played an important role in the rate of ovulation[19].

In a research on Sprague Dawley rats, similar dosage of present study was applied (30 IU of PMSG and 25 IU of hCG, and FSH alone). In comparison with our results, superovulation induction showed better response with PMSG[16,20]. There are also studies that FSH administration on immature Sprague Dawley rat resulted in higher number of counted corpora lutea than PMSG[18]. In addition, some researches showed that FSH had better effects on superovulatory response than PMSG, which was consistent with the results of this study. Moreover, it can be concluded that the possibility of implantation and fetal development using FSH treatment was greater than PMSG superovulatory protocols. The ability of these animals to have normal follicular development, ovulation and fetal growth demonstrates that FSH induces a physiologic conditions[21,22]. The weakness of PMSG response in normal development of embryos compared to FSH might because of three reasons: a delay of 26 h of primary ovulation after administration of PMSG[23], simultaneous activity of FSH and luteinizing hormone in PMSG preparations[5] and long half-life of PMSG[23]. However, in present study fertility of BALB/c mice was only observed in PMSG group.

Besides, injecting anestrous dog's serum in this study did not cause comparable superovulatory response with common hormones (PMSG in rat and FSH in mice). This may occur for two reasons. The first one is inhibitory effect of high serum prolactin levels in anestrous dog on the ovarian response to FSH hormone. In raw dog's serum, there is considerable amount of prolactin in addition to FSH. According to Olson *et al*[11], the concentration of prolactin is different in anestrus stage of various dogs. One of the six dogs in their experiment, the maximum concentration of prolactin was observed at late anestrus stage[11]. Therefore, the concentration of prolactin in serum samples may have been high in our study. This requires further investigation. Although presence or absence of prolactin in rodents has no effect on the rate of ovulation, maintaining corpora lutea and progesterone secretion, but it plays an important role in fertility continuance. On the other hand, Hyperprolactinemia suppresses gonadal function and reduces FSH and luteinizing hormone content in the blood of rodents[22,24]. Another reason for prevention of desirable superovulation may be the lack of purification and concentration of FSH hormone in collected samples of our anestrous dog's serum. Lack of purification means a lower presence of FSH concentration in the anestrous dog's serum needed for induction of satisfactory superovulatory response[11,25,26]. Although, the high concentrations of FSH have been shown previously in the blood of anestrous dog, however individual variations have been reported[11]. Furthermore, concentration of FSH during late anestrus was significantly higher than it during mid- and early anestrus. Accordingly, the effect of anestrous dog's serum on superovulation depends on individuals and phases of anestrous. In present study, serum of different dogs was mixed to overcome these variations and reach the same hormone concentration per volume. But, the concentration of FSH in injected serum may be less than required concentrations for induction of superovulation. This problem would be solved with FSH assay and purification procedures.

Intraperitoneal injection of anestrous dog's serum did not induce superovulation comparing to that of the PMSG in rats and rFSH in mice when corpora lutea number were counted 14 d after mating. This suggested that the serum FSH levels should be measured in anestrous dogs prior to administration.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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