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Development of Superovulation Program and Heterologous *in vitro* Fertilization Test Assessment in Hamsters

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ABSTRACT

Superovulation has become a common assisted reproductive technology in the field of animal reproduction. In addition, zona-free hamster oocytes have been used in heterologous *in vitro* fertilization research to evaluate sperm function. A study was conducted to compare eight different superovulation protocols for golden hamsters using two concentrations of human Chorionic Gonadotropin (hCG) given at two time intervals post-pregnant mare's serum gonadotrophin (PMSG) injection and two time intervals of oocyte harvesting. Fifty-six female golden hamsters were randomly and equally assigned into eight superovulation groups. Hamsters were superovulated initially with PMSG followed by human Chorionic Gonadotrophin (hCG). All the groups received 40 IU PMSG, either 40 or 45 IU hCG given at either 48-50 or 55-57 h post PMSG injection and the oocytes recovered at either 12-15 or 16-18 h after hCG injection. Higher number of recovered oocytes (51.57±0.83) and maturation rates (94.20%) ($p<0.05$) were detected in hamsters which received 45 IU hCG at 55-57 h after PMSG injection when the oocytes were recovered later at 16-18 h compared with hamsters in the other groups. Mean fertilization rate of hamsters given 45 IU hCG at 55-57 h post PMSG injection ranged from 77.89-78.84% and were significantly higher ($p<0.05$) than those that received hCG at 48-50 h post PMSG injection. In conclusion administration of 40 IU PMSG followed by 45 IU hCG injection at 55 and 57 h post PMSG injection followed by oocyte recovery after 16-18 h gave the highest response in oocyte recovery and maturation in golden hamsters.

Key words: Hamster, hCG, PMSG, uperovulation, zona-free hamster oocyte

INTRODUCTION

Superovulation has become a common technology in the reproductive management of farm and laboratory animals. This technique involves the induction of multiple ovulations during one oestrous cycle by administering exogenous hormones. Superovulation was first described by

Gates and Runner (1957) in mice and was later applied to other species, such as rat (Mukumoto *et al.,* 1995; Popova *et al.*, 2005; Kito *et al.*, 2010), mouse (Nagy *et al.*, 2003; Martin-Coello *et al.*, 2008; Luo *et al.*, 2011), rabbit (Treloar *et al.*, 1997; Kauffman *et al.*, 1998; Satheshkumar, 2006), hamster (Fleming and Yanagimachi, 1980; Roldan *et al.*, 1987; Bavister, 1989; Lee *et al.*, 2005), cattle (Kanitz *et al.*, 2002; Baruselli *et al.*, 2006), sheep (Ryan *et al.*, 1991; Grazul-Bilska *et al.*, 2007), pig (Hunter, 1964; Sommer *et al.*, 2007) and man (Market-Velker *et al.*, 2010).

Various gonadotropins have been used for superovulation. Equine chorionic gonadotropin (eCG) formerly known as Pregnant Mare Serum Gonadotropin (PMSG) has been used in laboratory animals to induce multiple ovulations before it was applied to livestock, namely cattle, buffalo and goats, to increase oocyte production. PMSG is a glycoprotein having both FSH and LH activities. Also, human chorionic gonadotropin (hCG), a glycoprotein hormone and similar in structure to Luteinizing Hormone (LH) which simulates the physiologic effects of LH, has been used to trigger the final follicular maturation before oocyte recovery in an ART program (Griesinger *et al.*, 2007).

The hamster has been used widely as a model for studying various diseases (Roberts *et al.,* 1978; Jorquera and Tanguay, 1997; Nonaka, 1998; Milazzo *et al.*, 2002). However, only a few protocols (Barnett *et al.,* 1997; Ludwig *et al.*, 2001; Lee *et al.*, 2005; Mckiernan *et al.*, 2005) have been developed for Assisted Reproduction Technologies (ART) in hamsters.

Yanagimachi *et al.* (1976) reported the first successful heterologous *in vitro* fertilization in golden hamsters. Since then, heterologous *in vitro* fertilization using hamster oocytes has been effectively pursued to predict male fertility in numerous species, including humans (Rogers *et al.*, 1979), cattle and horses (Brackett *et al.*, 1982). In several important aspects, the hamster *in vitro* fertilization system is one of the most useful models that had been used for the study of mammalian gamete interaction (Bavister, 1980). For the purpose of predicting male fertility, superovulation minimizes the number of females utilized to produce the required number of oocytes for gamete studies or any advanced embryological studies. Access to a reliable source of high quality harvested oocytes, with capability for fertilization and development, is an important requisite both for basic reproductive studies and applied research (Martin-Coello *et al.*, 2008).

In most hamster studies, it is tacitly assumed that collected and cultured oocytes and embryos from PMSG-stimulated females are 'normal'. However, there is increasing evidence that the quality of oocytes and embryos of these stimulated animals may be different from oocytes of non-stimulated females (McKiernan and Bavister, 1998). Therefore, the present study was conducted to establish an effective superovulatory program for golden hamsters in order to obtain a large quantity of high quality oocytes by manipulating the dose of hCG, time interval between PMSG and hCG injection and time interval between oocyte recovery and hCG injection.

MATERIALS AND METHODS

Experimental animals and design: Fifty-six pubertal female golden hamsters (*Mesocricetus auratus*) with body weight ranging from 120-150 g and age between 12 and 15 weeks old were purchased from Institute for Medical Research (IMR) in Kuala Lumpur, Malaysia. Prior to the start of the study the hamsters were adapted and maintained in a special room for two weeks under controlled lighting 12:12 h light-dark cycle, with the light switched on between 07:00 and 19:00 h. The room temperature was maintained at 22°C with approximately 60-70% humidity. These hamsters had exhibited at least two consecutive normal 4 day oestrous cycles, which were checked based on the vaginal discharge. The 56 golden hamsters were assigned

Table 1: Treatment groups of superovulation protocols with oocyte recovery time after hCG injection in the golden hamsters

Group[1]	PMSG (IU)	Interval between PMSG and hCG injection (h)	hCG (IU)	Oocyte recovery after hCG injection (h)
G1	40	48-50	40	12-15
G2	40	48-50	40	16-18
G3	40	48-50	45	12-15
G4	40	48-50	45	16-18
G5	40	55-57	40	12-15
G6	40	55-57	40	16-18
G7	40	55-57	45	12-15
G8	40	55-57	45	16-18

[1]n = 7 hamsters per group

randomly and divided equally into eight groups: G1 to G8 (Table 1). Superovulation was induced by intraperitoneal (i.p.) injection of 40 IU PMSG (Folligon® Intervet International, B.V. UK) in all groups regardless of the stage of estrus of the hamsters. Then, ovulation was induced by administration of either 40 or 45 IU hCG (Chorulon®; Intervet International, B.V. UK) injected intraperitoneally at 48-50 or 55-57 h after PMSG injection. Oocytes were then collected from the hamsters either 12-15 or 16-18 h after hCG injection.

Recovery of oocytes: Prior to oocyte recovery, the females were anesthetized with a combination of ketamine (80 mg kg^{-1} b.wt., Troy Laboratories PTY Limited, Australia) and xylazine (10 mg kg^{-1} b.wt., Troy Laboratories PTY Limited, Australia) following the recommendation of Heffner and Harrington (2002) intraperitoneally and then later sacrificed with an intracardiac injection of pentobarbital sodium 250 m kg^{-1} b.wt. (AKORN; Nembutal® Sodium Solution CII, USA). The oviducts were subsequently flushed with modified Tyrode's solution (TALP) for oocyte retrieval following the method described by Bavister and Yanagimachi (1977). After collection, the maturation status of oocytes was assessed by degree of cumulus expansion (De Loos *et al*., 1992), which was also used to assess oocyte quality. Expansion was characterized by the extremely sticky nature and enlargement of the cumulus mass to at least 0.5 mm in diameter (≥500 μm) away from the zona-pellucida. Lack of expansion which is an indication of poor-quality oocytes, was characterized by tight adherence of cumulus cells to the zona-pellucida. Nuclear maturation of oocytes was evaluated and confirmed by the formation of a first polar body extrusion examined under a stereoscopic microscope at 40×magnifications. This experimental procedure has been reviewed and approved by the animal care and use committee of Faculty Veterinary Medicine, Universiti Putra Malaysia (UPM/FPV/PS13.2.1.551/AUP-R106).

Removal of cumulus oophorus and zona pellucida: Oocytes collected from superovulated hamsters were treated with 0.1% (w/v) hyaluronidase (Type, I, H-3506; Sigma-Aldrich St. Louis, Missouri, USA) to remove the cumulus oophorus. The oocytes were then washed three times with wash-TALP medium. After washing the oocytes were transferred into a droplet containing 0.1% (w/v) trypsin (Type III, T-8003; Sigma-Aldrich St. Louis, Missouri, USA) placed in a petri dish to dissolve the zona pellucida. Digestion and dissolution of the zona pellucida were performed within 1 to 2 min and monitored under the dissecting microscope to avoid under or over treatment. Then, zona-free oocytes were washed three times with wash-TALP medium to remove any remnants of trypsin solution.

***In vitro* sperm capacitation:** Straws (0.5 mL) which contained frozen bull semen were thawed in a water bath at 38°C for 30 sec, pooled in a 15 mL Falcon tube (three straws per tube; each straw contained approximately 25-30×10^6 live sperm at the time of freezing) and diluted with 5 mL of Sperm-TALP solution. Then, the diluted bull semen was centrifuged at 350×g for 5 min motile sperm cells were isolated by the swim-up procedure as described by Lu *et al.* (1987). The final pellet obtained after the swim up was diluted in a capacitation medium (Sperm-TALP with 50 μg mL^{-1} heparin; Sigma No. H-5765) and acrosome reaction induced by adding 10 μM calcium ionophore A 23187 for 1 min (C-7522; Sigma, Aldrich Chemie GmbH, Germany) (Tardif *et al.*, 1999). The capacitated sperm pellet recovered after discarding the supernatant was diluted with the capacitation medium to give a sperm concentration of 2-4×10^6 sperm mL^{-1}. The sperm droplets (100 μL each) were prepared in a 35×10 mm petri dish covered with mineral oil and kept in an air incubator at 38°C.

Sperm-ocyte co-incubation, staining and penetration scoring: Between 10 and 15 washed zona-free hamster oocytes (ZFHOs) were placed in each sperm droplet (Fert-TALP) in a medium described by Bavister and Yanagimachi (1977) and incubated at 38°C for 3 h. After co-incubation, the ZFHOs were removed, washed and fixed on glass slides and stained with 1% Aceto-orcein, to examine sperm penetration. The presence of a distended sperm head, with a tail and male pronucleus was taken as an indication of successful sperm penetration, as described by Yanagimachi *et al.* (1976). Fertilization Percentage (FP) was calculated using the following formula:

$$\text{Fertilization}(\%) = \frac{\text{No. of ZFHOs penetrated by the spermatozoa}}{\text{No. of ZFHOs examined}} \times 100$$

Statistical analysis: Comparison between superovulation protocols on the number of oocyte recovered, maturation rate and fertihization percentage was done using one-way ANOVA, followed by a Duncan multiple range test. Differences between superovulation protocols were considered significant at $p<0.05$. The analysis was performed by SPSS (SPSS Inc. Version 20).

RESULTS

Oocyte recovery and maturation: The total number of oocytes recovered and maturation rate from hamsters in the eight different superovulation groups are shown in Table 2. In general,

Table 2: No. of oocytes harvested and maturation rate in golden hamsters following different superovulation protocols

Groups	Total No. of oocytes recovered per group[1]	Total No. of mature oocytes	Maturation rate (%)
G1	242 (34.57±0.84)^c	217 (31.00±0.87)^c	89.67^c
G2	252 (36.00±0.61)^c	231 (33.00±0.75)^c	91.67^c
G3	296 (42.28±0.68)^b	276 (39.42±0.64)^b	93.24^b
G4	310 (44.28±1.12)^b	291 (41.57±0.89)^b	93.87^b
G5	255 (36.42±0.84)^c	230 (32.85±0.80)^c	90.20^c
G6	261 (37.28±0.74)^c	240 (34.28±0.86)^c	91.95^c
G7	318 (45.42±0.57)^b	294 (42.00±0.53)^b	92.45^b
G8	363 (51.57±0.83)^a	342 (48.85±0.64)^a	94.20^a

[1]Data were expressed as total No. (Mean±SEM) from seven replicates (hamsters) per group, ^{abc}Values with different superscripts in the same column are significantly different at $p<0.05$

Table 3: Fertilization rate of oocytes fertilized with capacitated bull spermatozoa

Groups	No. of ZFHOs[1]	No. of penetrated ZFHOs	Fertilization rate (%)[2]
G1	217	154	70.95±0.91[b]
G2	231	161	69.94±2.35[b]
G3	276	203	73.58±1.98[ab]
G4	291	208	71.69±2.23[b]
G5	230	172	74.63±2.32[ab]
G6	240	167	69.73±2.38[b]
G7	294	227	77.23±1.39[a]
G8	342	270	79.01±2.05[a]

[1] ZFHOs: Zona-free hamster oocytes, [2] Data were expressed as mean±SEM from seven replicates [ab]Values with different superscripts in the same column are significantly different at $p<0.05$

groups which received higher doses of hCG (45 IU) showed higher number of oocytes recovered and subsequently, higher number of mature oocytes. Group 8 gave a significantly higher number of oocytes recovered and matured oocytes. In fact, among the eight groups, the highest number of oocytes recovered was obtained from hamsters given 45 IU hCG at 55-57 h after PMSG injection and when the oocytes were recovered 16-18 h later (mean of 51.57±0.83). Whilst the lowest number of oocytes was recovered from G1 with a mean of 34.57±0.84. Thus, the total number of oocytes recovered and the maturation rate of G8 was significantly highest ($p<0.05$) among the 8 superovulation groups. When comparison was made between 1-G6 groups which received equal doses of PMSG and hCG (40 IU each), there were no significant ($p>0.05$) differences observed in the mean number of oocytes recovered and maturation rate. These results indicated that when the time for oocytes recovery was delayed as in G2 and G6, the mean number of oocytes recovered slightly increased but not significantly different from G1 and G5 when the oocytes were recovered earlier at 12-15 h post hCG (G1; 34.57±0.84 vs. G2; 36±0.61 and G5: 36.42±0.84 vs. G6: 37.28±0.74).

Heterologous *in vitro* fertilization: Table 3 shows the results on heterologous *in vitro* fertilization of hamster oocytes with capacitated bull spermatozoa. The fertilization rate of G7 and G8 were significantly higher ($p<0.05$) than G1-G6 but not with G3 and G5 (Table 3). However, there was no significant difference ($p<0.05$) in fertilization rate between G7 and G8. The superovulation protocols for G7 and G8 resulted in similar fertilization rate achieved, 77.23 and 79.01%, respectively (Table 3).

DISCUSSION

The association of ovulation rate and days of oestrous cycle was inversely related with low ovulation rate found on days 3 and 4 of oestrous cycle in hamsters as reported by Fleming and Yanagimachi (1980). The results of the present study revealed that oocyte recovery and fertihzation rates were independent on the stage of the hamster's oestrous cycle. In the present study the optimum superovulation protocol which resulted in the highest mean number of oocytes recovered per female (51.57±0.83) and maturation rate (94.20) was achieved by the administration of 40 IU PMSG and 45 IU hCG at 55-57 h interval followed by oocyte recovery 16-18 h after hCG injection.

A previous study by Wilson and Zarrow (1962) showed dose of hCG, interval between PMSG and hCG injections and interval between hCG and recovery of the maximum number of ova

influenced the superovulatory response in immature mice and rats. The optimum concentration of PMSG for superovulation in hamsters was in the range of 30 to 45 IU as indicated by Greenwald (1974, 1976), Lee *et al.* (2005) and Kathiravan *et al.* (2008).

In the present study, the different groups of animals treated with equal doses of PMSG and hCG irrespective of the differences in the interval between PMSG and hCG injections and time of oocyte recovery generally showed insignificant differences in both the mean number of oocytes recovered as well as the maturation rate. This indicated that the time interval between PMSG and hCG injections and time of oocyte recovery was not a significant factor in influencing the variation in ovulatory rates. However, the optimum time interval between follicle stimulating substance (FSH) injection and the ovulatory dose of chorionic gonadotropin was reported to be 56 h in rats (Munalulu *et al.*, 1987), 40 h in mice (Fowler and Edwards, 1957; Gates and Runner, 1957) and 54-56 h in hamsters (Bodemer *et al.*, 1959). In the groups that were administered with 40 IU hCG, the number of recovered oocytes and mature oocytes was significantly lower than the 45 IU hCG groups. This indicates that the dose of hCG plays an important factor in influencing ovulatory rate. This finding is consistent with the earlier studies of Erickson and Shimasaki (2001), Grimmett and Perkins (2001) and Mehaisen *et al.* (2005). In addition, when comparisons were made among groups which were given the same dose of hCG, the number of oocytes recovered and the number of mature oocytes increased. These results are consistent with Wang *et al.* (2011) who reported that the percentage of mature oocytes can be improved by extending the interval between hCG and oocyte recovery.

The dose dependent superovulatory response of hamsters observed in this study is in agreement with Roldan *et al.* (1987) but the dosages that were used in the present study were about 3 times higher. Similar reports of using a combination of PMSG and hCG gave good response in other laboratory animals such as rats (Corbin and McCabe, 2002; Cornejo-Cortes *et al.*, 2006) and mice, (Martin-Coello *et al.*, 2008; Luo *et al.*, 2011). Corbin and McCabe (2002) reported that higher doses of PMSG (30 IU) and hCG (25 IU) increased significantly the number of oocytes recovered in female rats. Furthermore, according to Cornejo-Cortes *et al.* (2006), equal doses of PMSG (50 IU) and hCG (50 IU) produced the highest mean number of rat embryos when given 50 h apart. This was the highest PMSG: hCG dose combination attempted compared with other doses (30:30, 30:50, 50:30) in rats.

The effectiveness of PMSG injection in producing ovulated oocytes was extremely influenced by the stage of the oestrous cycle at which it was given (Lee *et al.*, 2005). Recovery rate was higher on days 3 and 4 of the oestrous cycle. Given 45 IU hCG, the hamsters in the current study assumed to be in late estrus showed significantly higher number of oocytes recovered compared with the other groups. Fleming and Yanagimachi (1980) reported that ovulation rate in golden hamsters was associated with the days of oestrous cycle, whereby ovulation rate decreased on days 3 and 4 of the oestrous cycle but increased on day 1. Thus in the present study it can be assumed that hamsters that had low ovulation rates were in days 3 and 4 of the ooestrous cycle.

In agreement with Fleming and Yanagimachi (1980) the present study indicated that, ovulation rate decreased on days 3 and 4 of the oestrous cycle compared with day 1 in golden hamsters following PMSG injection. The reduction in ovulation rate might be attributed to a deviation in gonadotropic properties among endogenous FSH and exogenous PMSG around day 4. Fleming and Yanagimachi (1980) concluded that extreme gonadotropin levels when PMSG was added on the normal FSH level subsequently might have an inhibitory influence on the development of follicles. The current optimum protocol comprising of the administration of 40 IU PMSG followed by 45 IU

hCG injection at 55-57 h later and subsequent oocyte recovery in 16-18 h was shown in the present study to result in highest number of oocytes recovered, leading to high maturation rate.

Although the superovulation protocol (40 IU PMSG, 45 IU hCG) in the present study produced the highest mean number of oocytes per female, maturation rate and fertilization rate, it doesn't guarantee in producing subsequent higher quality embryos compared with other protocols tested if the fertilized oocytes were subjected to culture or embryo-transfer. Hence further subcellular studies to evaluate the developmental competence of fertilized oocytes obtained by the different protocols compared with the optimum protocol should be able to confirm and identify the best protocol. According to Lee *et al.* (2005) although hamsters can be effectively superovulated by injecting equal doses of PMSG and hCG, a dose-dependent adverse effect on fertilized oocytes (embryos) development and sub-cellular microfilament distribution can occur.

In the present study, the ability to induce superovulation by the PMSG administration concurred with Roldan *et al.* (1987) which is in agreement with previous studies that reported the ability to retrieve good number of oocytes in Chinese hamsters and golden hamsters (Roldan *et al.*, 1987) as well as in other laboratory animals including rats (Hiroe *et al.*, 2005) and mice (Martin-Coello *et al.*, 2008). This has the advantage of minimizing the workload as it avoids the need to program superovulation by identifying animals on a particular stage (day 1) of the oestrous cycle to inject PMSG.

Heterologous *in vitro* fertilization test evaluated in the present study indicated higher fertilization rates from the golden hamsters given 45 IU of hCG. The extra follicles induced to ovulation by 45 IU hCG were physiologically normal as attested by the high percentage of fertilized oocytes. These findings implied that none of the doses of hCG and time interval tested inhibited growth and nuclear maturation of oocytes.

Generally, the present study was able to optimize the superovulation protocol in golden hamsters to obtain higher number of good quality oocytes. Though, the time interval between injection of PMSG and hCG and recovery interval of oocytes might have an impact on oocyte recovery, the dose of hCG appeared to be the most important factor in determining the superovulatory response of hamsters to the different superovulation protocols. The different interventions in the superovulation protocols on developmental competence of fertilized oocytes needs to be addressed in future studies.

CONCLUSION

In general, the present study succeeded to make effective the superovulation procedure for golden hamsters in order to obtain a lofty number of high-quality oocytes. Although the period between PMSG and hCG injections as well as recovery of oocytes might contribute to the rate of oocyte recovery, the hCG dose appeared to be the influential factor in determining the superovulatory response of hamsters to the different superovulation protocols. In conclusion, among the three factors examined in establishing the superovulation protocol for hamsters, hCG dosage is the most prominent factor in achieving the highest oocyte recovery rate. Higher dosage of hCG (45 IU) and longer interval between PMSG-hCG applications (55-57 h) and oocyte recovery (16-18 h) resulted in higher number of oocytes recovered, leading to higher maturation rate of the oocytes. Nevertheless, the different interventions in the superovulation protocols on developmental competence of fertilized oocytes needs to be addressed in future studies.

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