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Number and Quality of Oocytes Collected from Heterotopic Autografted Mice Ovary after PMSG Induction

NURBARIAH[1], ITA DJUWITA[1*], KUSDIANTORO MOHAMAD[1], IMAN SUPRIATNA[2]

[1]Department of Anatomy, Physiology and Pharmacology,Faculty of Veterinary Medicine, Bogor Agricultural University, Darmaga Campus, Bogor 16680, Indonesia
[2]Department of Clinical Reproduction and Pathology, Faculty of Veterinary Medicine, Bogor Agricultural University, Darmaga Campus, Bogor 16680, Indonesia

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Heterotopic grafting sites can be useful in producing oocytes for *in vitro* Fertilization, therefore, maximising the oocyte yield from the graft by gonadotrophin stimulation would be advantageous. The aim of this study was to investigate the number and quality of oocytes collected from heterotopic autografted ovary after Pregnant Mare Serum Gonadothropin (PMSG) induction. Graft recipients were treated either with or without PMSG stimulation 48 hours prior to graft collection. Ovarian tissue from four weeks old mice (DDY strain) were autotransplanted under the kidney capsule of the same ovariectomized mice and the oocytes were collected 21 days after autotransplantation. The results showed that the average number of oocytes collected from autografted ovaries without PMSG induction were 9.0. ± 2.8 not significantly different with those received PMSG induction, 10.9 ± 5.1. The percentage of matured and fertilized oocytes and the developed embryos from the autografted ovaries without PMSG induction were 52.4, 33.4, and 26.0%, respectively not significantly different with those received PMSG induction, 53.2, 35.1, and 29.9%, respectively. The number of oocytes and the capacity to matured, fertilized and developed were significantly lower ($P < 0.05$) compared to the superovulated nongrafted (control) ovaries. In conclusion, PMSG induction on the graft recipients did not significantly increase oocytes yield from grafted heterotopic ovaries. The number and quality of oocytes produced from the autografted ovaries were lower than the superovulated nongrafted ovaries, but still can be used for *in vitro* embryo production after sequential *in vitro* maturation and fertilization.

Key words: heterotopic autografted, ovary, PMSG induction, developmental potential

INTRODUCTION

Ovary as the primary organ in female reproductive system has two main roles i.e. for producing oocyte and synthesizing essential hormones for follicles development, estrous cyclicity and maintenance of the reproductive tract (Hirshfield 1991). Mammalian ovary contains large number of various developmental stages follicles. At the time of birth, cortex of mammalian ovary consist of abundant of primordial follicles as oocytes resources; those will develop during puberty (Fortune 1994). Ovaries collected from slaughter house or from immediately dead animal contain follicles in various development stages. These follicles are the oocytes resources used for embryo *in vitro* production (Gunasena *et al*. 1998; Shaw *et al*. 2000; Paris *et al*. 2004). This ovarian follicles will developed to produce development competence oocytes using *in vitro* culture and or grafting techniques (Eppig *et al*. 2000; Paris *et al*. 2004, 2009).

In the ovary, the oocytes developed simultaneously with the synthesizing and secreting hormones for supporting the follicles development and estrous cyclicity. However, in young woman having cancer, treatments such as irradiation and chemoterapy could lead to the loss of fertility. In order to protect this fertility, ovaries were ovariectomized and moved to safe area of the body through transplantation or cryopreseved (Eppig *et al*. 2000). After treatment, the cryopreserved ovary returned to the original sites (Hernadi *et al*. 2005). Ovary transplantation combined with *in vitro* culture techniques can be used for *in vitro* embryo production.

Transplantation is a method of transferring the organ or tissue to enable the growth of the transplant *in vivo*. Based on the correlation between donor and recipient, transplantation can be autotransplantation (transferring an organ from one site to the other sites of the same body); allotransplantation (transferring an organ from one individual to another individual of the same species) and xenotransplantation (of different species). Xenotransplantation is considered as a strategy for generating viable gametes that can be used to produce life fertile offspring of endangered species (Paris *et al*. 2004). Based on the sites, transplantation can be orthotopic (the same sites) or heterotopic (of the different site) such as subcutan and kidney capsule (Candy *et al*.

*Corresponding author. Phone: +62-251-8421823, Fax: +62-251-8629464, E-mail: djuwitawiryadi@yahoo.com

2000; Mohammad *et al*. 2004). Ovary heterotopic autografted is grafting the ovary in kidney capsule of the same body after being removed from the original site, the bursa ovary. The best site for heterotopic transplantation is the kidney capsule due to its high vascularization (Cox *et al*. 1996); however the number and size of the organ to be transplanted is very limited. Orthotopic grafts permit natural conception, while heterotopic grafts will required *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) for producing embryo (Gosden *et al.* 1994; Cox *et al.* 1996; Almodin *et al.* 2004a,b; Donnez *et al*. 2004).

Mouse ovary heterotopic autotransplantation in kidney capsule restore the estrous cyclicity on day 7 after transplantation and indicated the growth of follicles with normal morphology (Mohammad *et al*. 2004). Heterotopic ovarian grafts placed under the kidney capsule of mice yield viable oocytes and after *in vitro* fertilization resulted in pregnancy after the embryos were placed in recipient uterin (Carroll *et al.* 1990; Waterhouse *et al.* 2004).

The lifespan of a graft is limited because of the small size of the graft and follicle loss due to ischaemia after transplantation (Liu *et al.* 2002). Heterotopic grafting sites can be useful in producing oocytes for *in vitro* Fertilization (IVF); hence, maximising the oocyte yield from the graft by gonadotrophin stimulation would be advantageous. Based on the histological observation, superovulation treatment on the heterotopic autografted ovary showed an increased of tertiary follicles (Setiadi 2004). However, the number and quality of oocytes collected from fully antral follicles of those superovulated heterotopic autografted ovary need to be further examined, especially for the *in vitro* embryo production. Therefore, this research was aimed to examine the number and quality of oocytes collected from the fully antral follicles of heterotopic autografted ovary after Pregnant Mare Serum Gonadotropin (PMSG) induction.

MATERIALS AND METHODS

Experimental Animals. Four weeks old female of DDY strain mice were used as both ovarian tissue donors and graft recipients (n = 30). The graft recipient were grouped in two, group that stimulated with 5 IU PMSG (n = 10) and those without PMSG treatment (n = 10). Female mice of the same strain were treated with 5 IU PMSG and used for control for collection of *in vivo* matured (ovulated) oocytes (n = 10). The sperm donors were 12 weeks old male mice of the same strain. Intra peritoneal (i.p) injection of 5 IU PMSG was performed 48 hours before oocytes collection or on day nineteen after autotransplantation in the graft recipient PSMG treated group.

Heterotopic Autotransplantation. Each female mouse was used as both ovarian tissue donor and graft recipient in order to minimize the animal usage. Ovarian grafting was performed using standard procedures (Mohammad *et al*. 2004). Female mice were anaesthetized by administering each with 0.2 ml i.p. injection containing 1 mg/ml xylazine hydrochloride (Ilium Xylazil-20; Troy Laboratories, Smithtown, Australia) and 5 mg/ml ketamine hydrochloride (Ketamine; Parnell Laboratories, Alexandria, Australia). Once anaesthetized, the dorsal skin of both mice was swabbed with 70% (v/v) alcohol and a 1 cm dorsolateral incision was made through the skin and peritoneum to expose the lipid pad with ovary attached to it and kidney. Using fine forceps the lipid pad was pulled out from the abdomen cavity. The lipid pad was clip using serrefine clamp and the ovary was separated from the bursa. The excised ovaries were placed in Dulbecco's Phosphate Buffered (PBS; Gibco BRL, USA) and were cut in half before being grafted. Bilateral ovariectomy was performed on each mouse. A small incision was made into the kidney capsule and two hemi-ovaries were inserted under each kidney capsule. The skin was closed with cotton suture.

Pregnant Mare Serum Gonadotrophin (PMSG) Induction (Hogan *et al*. 1986). Nineteen days after grafting, recipients were divided into two groups as follows: recipients receiving PMSG (n = 10) and non-receiving PSMG recipients (n = 10). Mice that were treated with PMSG were given a single i.p. injection of 5 IU PMSG (Folligon; Intervet, Australia). Three weeks after grafting, all graft recipients were killed by cervical dislocation. The control group did not receive any ovarian grafts (n = 10); these mice were superovulated with an i.p. injection of 5 IU PMSG (Folligon) followed 48 h by an i.p. injection of 5 IU hCG (Chorulon) and allowed *in vivo* oocytes maturation and ovulated.

Oocyte Collection and *In Vitro* Maturation. The grafts were collected into PBS medium at 37 ^{o}C. The oocytes were released by puncturing the large (fully) antral follicles with 26 G needle. At the time of collection, oocytes were fully cumulus enclosed (tight cumulus cells) for which the nuclear status could not be assessed. All oocytes were cultured in 20-30 µl droplets of kalium simplex optimized medium (KSOM) supplemented with 10 µg/ml follicle stimulating hormone (FSH), 50 µg/ml gentamycin sulphate and 3% (w/v) Bovine Serum Albumine (BSA) covered with mineral oil and incubated for 24 h at 37 ^{o}C, 100% humidity and 5% CO_2 in air. Throughout the experiments 35 mm Falcon culture dishes (Becton Dickinson, Franklin Lakes, NJ, USA) were used.

In control group, the ovulated oocytes were collected 13-14 h after the hCG injection to allow insemination at the same time as the oocytes from the ovarian grafts. The oviducts were removed and placed into KSOM medium and the cumulus mass released by puncturing the fertilization vesicle of the ampulla.

The number of oocytes collected per graft was noted. Prior to maturation status evaluation, oocyted from all groups were denuded with 0.1% hyaluronidase for 10 min to remove the cumulus cells. Matured oocyte was indicated by the presence of first polar body (PB I) or the absence of Germinal vesicle (GV). Matured oocytes were determined by the presence of PB I.

Sperm Preparation. Male mice were killed by cervical dislocation and the testes removed. The cauda of the epididymis were dissected, cut into strips and each cauda placed into 1 ml KSOM in 6 ml Falcon tubes (Becton

Dickinson). The sperm were incubated in 5% CO_2 incubator at 37 °C, 100% humidity, for 30-60 min. The insemination drops were then prepared by placing 30 µl aliquots of this medium containing the sperm into 35 mm Falcon dishes and then covering them with pre-equilibrated mineral oil.

In Vitro **Fertilization and Embryo Culture.** The oocytes were washed in three 20 µl preincubated droplets of KSOM medium before being placed into a droplet containing sperm. Six hours later the oocytes were washed three times in preincubated 20 µl droplets of KSOM medium supplemented with 50 µg/ml gentamycin sulphate and BSA (fraction V; Bayer, West Haven, CT, USA) and then cultured for 4 days. Fertilization of the oocytes from the ovarian grafts was performed at the same time as the collection and fertilization of ovulated oocytes from the oviducts of (superovulated) age-matched mice of the same strain. These metaphase II (MII) oocytes served as controls were therefore fertilized with the same sperm as the oocytes that were collected from the ovarian grafts.

Experimental Design and Data Analysis. The percentage of matured and fertilized oocytes were calculated from the number of oocytes showed PB I and 2 PN, respectively, per the number of GV oocytes x 100. The percentage of cleavage embryos were calculated from the number of embryos at the 2 to 8 cells stages per the number of GV oocytes cultured x 100. Data were analized using general linear method by Duncan's multiple range test. Statistical significance was established at the $P < 0.05$ level. The statistical analysis was performed on the SPSS 17.0 program.

RESULTS

Ovarian grafts were successfully recovered from all graft recipients in both without and with PMSG stimulation, and showed of fully antral follicles development (Figure 1a,b). As grafts at heterotopic site do not allow spontaneous conception, the oocytes have to be harvested from the fully antral follicles for further *in vitro* maturation (IVM) and fertilization (IVF). The average number of oocytes collected from a pair of grafted ovaries without and with PMSG stimulation were 9.0 ± 2.8 and 10.9 ± 5.1, respectively ($P > 0.05$). The average number of oocytes from the grafted ovaries were significantly lower compared with the superovulated nongrafted ovaries (as control) (20.0 ± 4.4) (Table 1). In contrast to the control nongrafted ovaries, the number of oocytes recovered from heterotopic autografted ovaries did not increase after PMSG stimulation. And the overall average numbers of oocytes from the grafted ovaries were significantly lower compared with the superovulated nongrafted (control) ovaries.

All oocytes collected from antral follicles of the grafts recipients treated with PMSG were enclosed with tight compact cumulus cells similar to the non PMSG stimulated recipients (Figure 2a). This tight enclosed-cumulus cells indicated that the oocytes have not reached the matured stage and examination after hyaluronidase treatment (nude oocytes) under phase contrast microscope showed that they were at the Germinal Vesicle (GV) stage (Figure 2b). After *in vitro* maturation, the tight enclosed cumulus cells showed extensive expansion (Figure 2c) and the matured oocyte was determined by the presence of the first polar body or the absence of the GV (Figure 2d). The percentage of matured oocytes from the grafted ovaries without PMSG stimulation was 52.4%, was not significantly different with those that received PMSG stimulation (53.2%); while the percentage of matured oocytes from the control nongrafted ovaries was 84.4%, significantly higher than those from the grafted ovaries (Table 2).

As the matured oocytes were inseminated, the sperm will progressively move through the expanded cumulus and penetrated into the zona pellucida and vitelline membrane (Figure 2e). The penetration of sperm will activate the oocyts to resumes the second meiosis and extruded the second polar body (Figure 2f). The percentage of fertilized oocytes (determined by the presence of 2 pronuclei, Figure 2g) from grafted ovaries without and with PMSG stimulation were 33.4 and 35.1%, respectively ($P > 0.05$), signifantly lower than of the control nongrafted ovaries (64.4%).

The percentage of cleavage embryos were 31.0 and 29.9% ($P > 0.05$) in grafted without and with PMSG stimulation, respectively; but significantly lower compared to those with the superovulated nongrafted ovaries 60.0%.

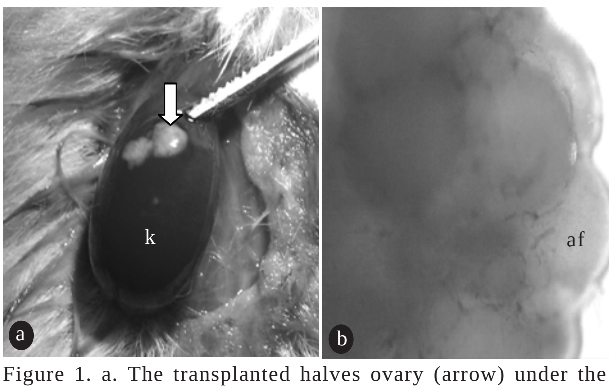

Figure 1. a. The transplanted halves ovary (arrow) under the kidney (k) capsula; b. The grafted ovary after removed from the kidney capsule, showed the developed antral follicles (af).

Table 1. Average number of oocytes collected from heterotopic autografted ovary with and without PMSG induction

Ovary treatment	No. of ovary	Mean ± SD per mouse	Mean ± SD per ovary
Autografted, no PMSG	10	9.0 ± 2.8a	4.5 ± 1.4a
Autografted, received PMSG	10	10.9 ± 5.1a	5.5 ± 2.6a
Nongrafted, received PMSG	10	20.0 ± 4.4b	10.0 ± 2.2b

Within coloumn a,b significantly different $P < 0.05$.

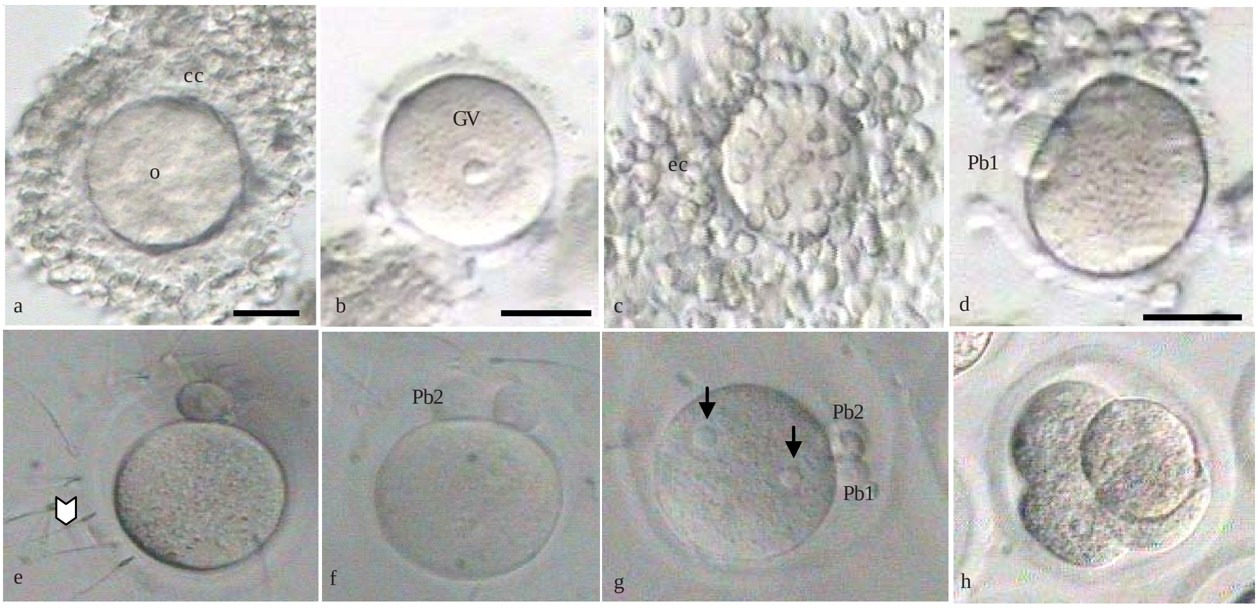

Figure 2. Oocytes collected from antral follicles of the grafts recipients treated with PMSG a. Oocyte (o) with compact cumulus (cc) isolated from the antral follicle; b. Oocyte at the GV stage; c. Oocytes with expanded cumulus (ec) cells after *in vitro* maturation; d. Oocyte at the metaphase II stage, the presence of the first polar body (pb1); e. Matured oocytes inseminated with sperm (arrowhead); f. Extrusion of the second polar body after oocyte insemination; g. Fertilized oocyte with 2 pronuclei (arrow) and second polar body (pb2); h. Cleavage embryo at 4 cells stage. Bar: 50 μm (a,c,e); (b,f,g); (d,h).

Table 2. The percentage of oocytes *in vitro* maturation and fertilization and the embryo development

Ovary treatment	GV oocytes	Number of (%)		
		Matured (MII) oocytes	Fertilized oocytes	Cleavage embryo
Autografted, no PMSG	84	44 (52.4)a	28 (33.4)a	26 (31.0)a
Autografted, received PMSG	94	50 (53.2)a	33 (35.1)a	28 (29.9)a
Nongrafted, received PMSG	160	135 (84.4)b	103 (64.4)b	96 (60.0)b

Percentages are expressed as number of matured or fertilized oocytes or cleavage embryos per total GV oocytes. Within coloumn a,b significantly different $P < 0.05$.

DISCUSSION

The number of oocytes recovered from the fully antral follicles of heterotopic grafted ovaries with and without PSMG induction was not significantly different; this showed that PMSG induction does not significantly increase the oocyte yield from ovarian grafts under the kidney capsule. This result was in line with the previous report in mice, that administration of exogenous gonadotrophins does not significantly increase the oocyte yield from ovarian grafts under the kidney capsule (Waterhouse *et al.* 2004; Yang *et al.* 2006). And similar result has also been reported that heterotopic mouse ovarian xenografts failed to produce an increase in oocyte yield in response to exogenous PMSG treatment (Snow *et al.* 2002).

In contrast, the number of oocytes collected from nongrafted ovaries were significantly higher compare to the PSMG stimulated heterotopic grafted ovaries. However, Waterhouse *et al.* (2004) reported that superovulation treatment using PMSG on orthotopic grafted ovaries also did not increase the oocytes yield. This might be due to hormonal feedback between the pituitary and ovary that regulates oocyte development and may be disturbed by grafting and in particular grafting to heterotopic sites. The kidney capsule graft site has a relatively high vascularization that facilitates the revascularization process which is essential for graft establishment and the support of follicular growth (Cox *et al*. 1996). However, the revascularization process depends on several factors, such as the size of the tissue, the graft site and the presence of angiogenic factors. In mouse, with relatively small size ovaries, vascularization might establish within 24-48 h of grafting (Dissen *et al*. 1994). Prior to revascularization or post-transplant, the transplant ovaries could undergo ischaemia that could cause for the graft and follicles lost. As reported by Israely *et al*. (2006), in mice, transplantation accounts for approximately 42% of the loss in follicle population.

All oocytes collected from antral follicles of the grafted ovaries (without and with PSMG induction) were enclosed with tight compact cumulus cells (Figure 2a), indicate that the oocytes have not reached the matured stage and examination after hyaluronidase treatment (nude oocytes). Under phase contrast microscope it was showed that they were at the Germinal Vesicle (GV) stage (Figure 2b). Gonadotropin stimulation in this research was performed by administration of PMSG without hCG as the hormone responsible for the GV breakdown. However, Carroll *et al.* (1990) and Waterhouse *et al.* (2004) collected oocytes from ovarian grafts on the kidney 12 h after hCG injection showed that the majority of the oocytes were at the GV stage. This results may indicate that the follicular/oocyte response to exogenous gonadotrophins at a heterotopic site may not be normal. The reason for this is still unknown, but it might be possible that the vascular remodeling that occurs in the ovarian graft does not allow adequate delivery of the gonadotrophin to the grafts.

This study examined the developmental potential of oocytes collected from heterotopic autografted ovaries (at the kidney capsule). For producing embryo for conception, oocytes collected from heterotopic grafts required *in vitro* maturation (IVM) and *in vitro* fertilization (IVF). Oocytes collected from ovarian grafts appear to have a reduced developmental potential in comparison to the oocytes produced from nongrafted ovaries. The developmental competence (both the IVM and IVF) of oocytes was lower in all the graft groups compared to the superovulated oocytes from nongrafted ovaries. This might be, in part, due to the oocytes from grafts undergoing *in vitro* maturation or an effect of grafting itself. The grafting process causes ischemic-reperfusion injury that occurs as the graft establishes a new blood supply. This period of poor vascular support may adversely affect the oocyte and growing follicles. Damage to the perivascular and endothelial cells was influence to the integrity of ovarian follicles and oocytes in ovarian grafts (Israely *et al*. 2006). Ovarian grafting also disrupts innervation of the ovary. The ovary is innervated by extrinsic and intrinsic nerves that are believed to control blood flow, as well as follicle and oocyte development (Anesetti *et al*. 2001; D'Albora *et al*. 2002; Aguado 2002). Normal follicle development and the production of viable oocytes is a complicated process that involves coordination of numerous molecules including hormones, growth factors and receptors, in various signaling pathways that act in autocrine, paracrine and endocrine manners (Yamashita *et al.* 2000; Josefsberg & Dekel 2002; Drummond *et al.* 2003). Although grafted ovaries do become revascularized and reinnervated, the grafting process may perturb the systems that are essential for normal follicle growth and oocyte development and thus result in a reduced number of developmentally competent oocytes.

Oocytes from grafts in heterotopic groups are deprived of local factors from the uterus or orthotopic site that are required for normal follicle and oocyte development. Furthermore, transplantation of ovarian tissue to other sites within the body may disrupt some ovarian processes and/or expose the ovary to a different environment.

Pregnant Mare Serum Gonadotropin (PMSG) stimulation on heterotopic grafted ovaries did not increase the number and quality of oocytes produced. The development competence of oocytes produced from the heterotopic autografted ovaries was lower than those from stimulated nongrafted ovaries. Heterotopic autografted ovaries produced oocytes that can be used for *in vitro* embryo production after matured and fertilized *in vitro*.

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