GH Increases the Progesterone at Peri-estrus Stage in Mice Co-injected with PMSG for Superovulation

Young Gee Kim, Zae Young Ryoo and Young Sik Park†

School of Animal Science and Biotechnology, Kyungpook National University, Sangju 742-711, Korea

ABSTRACT

Growth hormone (GH) is obligatory for growth and development. But, there is controversy on the GH effect about reproductive processes of sexual differentiation, pubertal maturation, gonadal steroidogenesis, gametogenesis and ovulation. This study was conducted to investigate the effect of GH on estrus, ovulation and embryo implantation. The results obtained were as follows. GH stimulated to increase estrus rate ($p<0.05$), pregnancy rate ($p<0.05$), and total fetus number in mice treated for superovulation. Also, the correlation between GH and steroids, E2 and P4, at peri-estrus stage/ peri-ovulation stage/ peri-implantation stage of the superovulation-induced mice was examined. Consequently, GH co-injected with PMSG especially increased P4 level ($p<0.05$) at peri-estrus stage of superovulation-induced mice. In conclusion, GH co-treatment in superovulation system boosted the rate of estrus, pregnancy and total fetus by increasing progesterone level at peri-estrus stage of superovulation-induced mice.

(Key words : GH (Growth hormone), E2 (Estradiol-17β), P4 (Progesterone), Estrus, Pregnancy)

INTRODUCTION

Growth hormone (GH) is generally released from pituitary somatotrophs into the circulation which acts at distant target sites to promote cell growth and differentiation. These actions may reflect direct endocrine actions of pituitary GH or be mediated by its induction of hepatic or local IGF-I production. However, as GH is also produced in gonadal, placental and mammary tissues, it may act in paracrine or autocrine ways to regulate local processes that are strategically regulated by pituitary GH. de Ziegler *et al.* (2011) reviewed that co-treatment with GH improved assisted reproduction outcome in poor controlled ovarian stimulation responders through meta-analyses.

Superovulation is ovarian hyperstimulation for the release of more eggs by injecting gonadotropic hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH), for emrbyo transfer. FSH stimulates the selection and growth of ovarian follicles by synthesis and secretion of estradiol 17 beta (E2) form granulosa cells and LH induces the ovulation of the matured follicles by synthesis of progesterone (P4) from thecal cells. E2 and P4 are essential for the growth and function of female reproductive organ and tract and for follicular recruitment, oocyte maturation, ovulation, corpus luteal function, the maintenance and implantation of the blastocyst and the regulation of the hypothalamo-pituitary-gonadal axis.

Although LH and FSH are the primary regulators of ovarian steroidogenesis, GH may also modulate the production of ovarian steroids. Wilson *et al.* (1991) reported that GH levels were modestly increased during the follicular phase of the normal menstrual cycle, but the levels were markedly increased during superovulation with hMG or hMG plus GnRH agonists, and estradiol levels were parallel increased. However, the estradiol level increased during superovulation may modify the oviductal environment, resulting in higher rates of unfertilized ova or degenerated embryos.

There is controversy on the GH effect about reproductive performance. Cecim *et al.* (1995) reported that sexual maturation was accelerated in phosphoenolpyruvate carboxykinase (PEPCK)/bGH transgenic females. And Danilovich *et al.* (1999) insisted that GH resistance and consequent reduction in peripheral IGF-I levels shown in mice homozygous for targeted disruption of the GH receptor/GH binding protein gene was associated with delay of female puberty, alterations in fetal and placental growth, delay of parturition, and reduction of litter size. On the other hand, Folch *et al.* (2001) reported that the application of pGH (porcine Growth Hormone) to superovulated ewes did not significantly affect the percentage of ewes in estrus, the time of estrus onset or the ovulation rate.

In order to settle down the controversy and elaborate the action mechanism of GH for the reproductive functions, this study investigated the effect of GH on the induction of estrus and pregnancy by analysing the

† Corresponding author : Phone: +82-054-530-1223, E-mail: yspark@knu.ac.kr

levels of steroids, estradiol-17β (E2) and progesterone (P4), in sera of superovulation-induced mice.

MATERIALS AND METHODS

Experimental Animals and Housing Conditions

Female FVB mice of 7 weeks old were allocated at random and housed under the specific pathogen-free (SPF) condition in the room controlled as follows; temperature of 22±1℃, ammonia concentration of below 20 ppm, noise level of below 60db, illumination of 200~300 lux and relative humidity of 50±5%. Lighting was adjusted automatically to a cycle of 12 hours light and 12 hours dark. The room air was automatically ventilated 15 times per hour. For animal health care, the autoclaved cages and beddings were used and the irradiated feed (Harlen, Madison, USA) and sterilized water were supplied free to access.

All experiments were conducted according to the Kyungpook National University guidelines for the care and use of laboratory animals.

Superovulation Treatment

Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were purchased from Sigma chemical Co. (St. Louis, MO, USA) and used as the substitutes of FSH and LH for superovulation, respectively. Female mice were superovulated by injecting interperitoneally with 5 IU of PMSG at 4:00 p.m. of Day 1 and with 5 IU of hCG at 4:00 p.m. of Day 3, forty-eight hours after the PMSG injection. Immediately after hCG injection, the female was transferred and housed in male cage with the fertile male. Ovulation may occur approximately 12 hours after the hCG injection, at which time the eggs can be fertilized.

GH Treatment

Recombinant human Growth hormone (GH, Eutropin) was provided by LG Life Science (Seoul, Korea). Details of the somatotropin are as follows; recombinant, (Vector) pYLBC ADH/GAP hGH, (Host) *Saccharomyces cerevisiae* 2150.

GH was diluted with PBS before use and 0.3 IU of GH per 20g of BW was intraperitoneally co-injected with PMSG into female mice for analysing the effect of GH on superovulation induction.

Estrus Evaluation

The females co-housed with male was checked for vaginal plug at 8 a.m. of next morning after hCG injection. The vaginal plug should be an estrus marker at natural mating because it was consisted of coagulated proteins of the male seminal fluid. It was possible that females often will become pregnant when no plug has been observed. But, the vaginal plug in most mouse strains can be easily observed approximately 12 to 14 hours after mating.

Pregnancy Evaluation

The female mice were anesthetized at 4.5 day after hCG injection (peri-implantation stage) with both 100 μg of Ketamine (Yuhan, Co., Korea) and 10 μg of Xylazine (Bayer Korea Co., Korea) per 1g of body weight (BW). After recovering and washing the uteri from the anesthetized females with phosphate buffered saline (PBS), fetuses were immediately counted in the open uterus.

Analysis of Serum P4 and E2

Blood sample was taken from infraorbital plexus using the heparin-coated capillary tubes (I.D.; 1.1~1.2 mm, length; 75 mm). It was centrifuged at 3,000 rpm for 10 minutes to separate serum. The serum was stored at −80℃ until use.

The serum was recovered 1) at 0.5 days before injection of hCG, so called as peri-estrus stage, 2) at 0.5 day after injection of HCG, so called as per-ovulation stage, 3) at 4.5 days after injection of hCG so called as peri-implantation stage, respectively.

The serum recovered was used for analysis of the levels of E2 and P4 by Radioimmunoassay (RIA) using reagents purchased from Diagnostic Products Corporation (CA. USA). Analysis of E2 and P4 were performed by E-ONE medical center (Seoul, Korea). The sensitivity of E2 and P4 assay were blow 10 pg/ml and 0.02 ng/ml, respectively.

Statistical Analysis

Statistical analysis was performed with a package programs using one way analysis of variance (ANOVA) and χ^2-test (MICROSTAT, Ecosoft Inc.) and one sample T test (dBSTST 4 for Windows, Analyse-It Software Ltd.). A value of $p<0.05$ was accepted as an indication of statistical significance.

RESULTS

GH and Reproductive Traits in Superovulation-Induced Mice

The reproductive traits, estrus and pregnancy, in the mice co-treated with GH were examined and the result obtained was shown in Table 1.

In the experiment to examine the effect of GH co-treatment for the expression of reproductive traits, the estrus rates of PMSG and PMSG+GH groups were 64.6 ±2.1% and 87.5±2.4%, respectively and the pregnancy ra-

Table 1. Effect of GH co-treatment on estrus and pregnancy in superovulated-mice

Treatment	No. of mice used (A)	No. of mice with virginal plug(B)	Estrus rate* (%, B/A)	No. of mice with fetus(C)	Pregnancy rate* (%, C/A)
PMSG	48	31	64.6±2.1[b]	29	60.4±2.1[b]
PMSG+GH	48	42	87.5±2.4[a]	38	79.2±5.4[a]

*; Four sets of replications; Each value represents mean±SE.
[a,b]: Significantly different in the same column (p<0.05).

tes of PMSG and PMSG+GH groups were 60.4±2.1% and 79.2±5.4%, respectively. This results indicated that GH significantly increased the estrus expression and pregnancy induction in superovulation-induced mice.

GH and Serum E2 and P4 Levels in Superovulation-Induced Mice

The previous results also suggest that GH may stimulate the steroid hormones, E2 and P4, by considering the increased estrus and pregnancy rates in mice co-treated with PMSG and GH. Carlsson *et al.* (1992) have reported that GH augmented basal and/or follicle stimulating hormone-stimulated steroidogenesis in granulosa cells where functional growth hormone receptors were found.

GH and Steroids at Peri-Estrus Stage

The levels of steroids, E2 and P4, at peri-estrus stage of the mice co-treated with GH was examined by RIA in sera recovered at 0.5 days before hCG injection and the result obtained was shown in Table 2.

In the experiment to examine the effect of GH co-treatment for the expression of steroids at peri-estrus stage, the E2 levels of PMSG and PMSG+GH groups were 33.35±5.1 and 39.22±6.7 pg/ml, respectively, the P4 levels of PMSG and PMSG+GH groups were 7.25±10.13 and 14.30±0.7 ng/ml, respectively, and the E2/P4 ratios of PMSG and PMSG+GH groups were 0.467±0.085 and 0.274±0.045, respectively. That is, at peri-estrus stage of superovulation-induced mice, GH increased less 17.6% of E2 level from 33.35±5.1 to 39.22±6.7 pg/ml and more 97.2% of P4 level from 7.25+0.13 to 14.30±0.7 ng/ml.

The results indicated that GH co-treated with PMSG did not significantly increase the E2 level, but significantly increased the P4 level, and that GH significantly decreased the E2/P4 ratio by increasing less E2 and more p4 levels at peri-estrus stage. Therefore, GH co-treatment especially increased P4 level at peri-estrus stage of superovulation-induced mice.

GH and Steroids at Peri-Ovulation Stage

The expression of steroids, E2 and P4, at peri-ovulation stage of the mice co-treated with GH was examined by RIA in sera recovered at 0.5 days after hCG injection and the result obtained was shown in Table 3.

In the experiment to examine the effect of GH co-treatment for the expression of steroids at peri-ovulation stage, the E2 levels of PMSG and PMSG+GH groups were 19.91±1.7 and 15.92±2.4 pg/ml, respectively, the P4 levels of PMSG and PMSG+GH groups were 12.60±3.5 and 15.71±1.4 ng/ml, respectively, and the E2/P4 ratios of PMSG and PMSG+GH groups were 0.179 ±0.044 and 0.101±0.013 respectively. That is, at peri-ovulation stage of superovulation-induced mice, GH co-treatment decreased 20.0% of E2 level from 19.91±1.7 to 15.92±2.4 pg/ml and increase 24.7% of P4 level from 12.60±3.5 to 15.71±1.4 ng/ml.

The results indicated that GH co-treated with PMSG did not significantly decrease the E2 level and increase the P4 level, and that GH did not significantly decrease the E2/P4 ratio by decreasing E2 level and increasing P4 level at peri-ovulation stage. Therefore, GH co-treatment did not significantly affect the E2 and P4

Table 2. Effect of GH co-treatment on E2 and P4 levels in mouse serum recovered at 0.5 days before hCG injection

Treatment	E2 (pg/ml)*	P4 (ng/ml)*	E2/P4 ratio*
PMSG	33.35±5.1[NS]	7.25±0.13[a]	0.467±0.085[a]
PMSG+GH	39.22±6.7	14.30±0.7[b]	0.274±0.045[b]

*; Three sets of replications; Each value represents mean±SE.
[NS]: Not Significantly different from each group in the same column (p>0.05).
[a,b]: Significantly different in the same column (p<0.05).

Table 3. Effect of GH co-treatment on E2 and P4 levels in mouse serum recovered at 0.5 days after hCG injection

Treatment	E2(pg/ml)*	P4(ng/ml)*	E2/P4 ratio
PMSG	19.91±1.7[NS]	12.60±3.5[NS]	0.179±0.044[NS]
PMSG+GH	15.92±2.4	15.71±1.4	0.101±0.013

*; Three sets of replications; Each value represents mean±SE.
[NS]: Not Significantly different from each group in the same column (p>0.05).

Table 4. Effect of GH co-treatment on E2 and P4 levels in mouse serum recovered at 4.5 day after hCG injection

Treatment	E2(pg/ml)*	P4(ng/ml)*	E2/P4 ratio
PMSG	19.61±2.6^{NS}	39.24±0.8^{NS}	0.050±0.006^{NS}
PMSG+GH	16.10±1.1	34.42±5.6	0.051±0.014

*; Three sets of replications; Each value represents mean±SE.
NS: Not Significantly different from each group in the same column (p>0.05).

levels at peri-ovulation stage of superovulation-induced mice.

GH and Steroids at Peri-Implantation

The expression of steroids, E2 and P4, at peri-implantation stage of the mice co-treated with GH was examined by RIA in sera recovered at 4.5 days after hCG injection and the result obtained was shown in Table 4.

In the experiment to examine the effect of GH co-treatment for the expression of steroids at peri-implantation stage, the E2 levels of PMSG and PMSG+GH groups were 19.61±2.6 and 16.10±1.1 pg/ml, respectively, the P4 levels of PMSG and PMSG+GH groups were 39.24±0.8 and 34.42±5.6 ng/ml, respectively, and the E2/P4 ratios of PMSG and PMSG+GH groups were 0.050±0.006 and 0.051±0.014, respectively. That is, at peri-implantation stage of superovulation-induced mice, GH decreased 17.9% of E2 level from 19.61±2.6 to 16.10±1.1 pg/ml and 12.3% of P4 level from 39.24±0.8 to 34.42±5.6 ng/ml.

The results indicated that GH co-treated with PMSG did not significantly decrease the E2 and the P4 levels, and that GH did not significantly decrease the E2/P4 ratio by decreasing E2 and P4 levels at peri-implantation stage. Therefore, GH co-treatment did not significantly decreased the E2 and P4 levels at peri-implantation stage of superovulation-induced mice.

Comparison of PMSG and PMGS+GH superovulation systems

The E2 and P4 levels at peri-estrus, at peri-ovulation and at peri-implantation stages of the mice coinjected with GH and/or PMSG were examined by RIA in sera recovered at 0.5 days before, at 0.5 days after and at 4.5 days after hCG injection, respectively, and the results obtained were shown in Fig. 1.

E2 levels at peri-estrus stage was higher than those at peri-ovulation and peri-implantation stages in both of PMSG and PMSG+GH systems. However, P4 levels in PMSG system continuously increased from peri-estrus stage, to peri-ovulation stage and to peri-implantation stage, but, P4 levels in PMSG+GH system showed high at peri-estrus stage as much as at peri-ovulation stage and increased highest at peri-implantation stage. Therefore, new PMSG+GH superovulation system resulted in higher estrus and pregnancy rates by increasing progesterone level at per-estrus stage compared to conventional PMSG superovulation system.

GH and Fetus Number in Superovulated Mice

The previous results showed that GH increased estrus and pregnancy rates by affecting levels of steroid hormones, E2 and P4, especially by increasing P4 at peri-estrus stage in mice co-injected with PMSG. This result suggested that GH may affect ovulation and em-

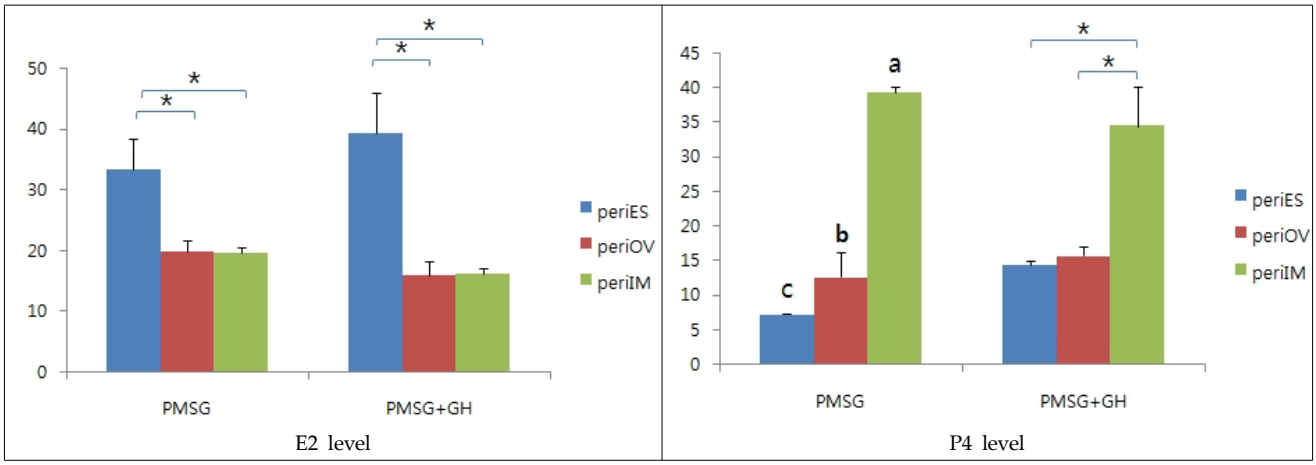

Fig. 1. E2 and P4 levels in mice co-injected with GH and/or PMSG. They were analysed by RIA in sera recovered at 0.5 days before, at 0.5 days after and at 4.5 days after hCG injection, respectively. The E2 levels at three stages showed a similar pattern and P4 levels at three stages showed a different pattern between PMSG and PMSG+GH groups. GH co-treated with PMSG early increased the serum P4 level at peri-estrus stage. The data represent mean±SE (n=3). periES; peri-estrus stage, periOV; per-ovulation stage, periIM; peri-implantation stage. * p<0.05.

Table 5. Effect of GH on fetus number in superovulation-induced mice

Treatment	No. of mice used*	Total no. of fetuses obtained	Mean no. of fetuses per mouse
PMSG	48	540[b]	11.25[b]
PMSG+GH	48	592[a]	12.33[a]

*: Four sets of replications; Each value represents mean±SE.
[a,b]: Significantly different in the same column (p<0.05).

bryo development in superovulation-induced mice.

The ovulation and embryo development are closely related to fetus number that is an important objective of embryo transfer. Thus, the fetus number was examined in the mice superovulated with GH and the result obtained was shown in Table 5.

The total number of fetuses in PMSG and PMSG+GH groups were 540 and 592, respectively, and the mean number of fetuses in PMSG and PMSG+GH groups were 11.25 and 12.33, respectively. These results indicated that GH may affect the ovulation and embryo development by considering the fetus number in superovulated-mice. Therefore, GH treatment in superovulation system boosted the rate of estrus, pregnancy and total fetus by increasing progesterone level at peri-estrus stage of superovulation-induced mice.

DISCUSSION

GH and Estrus in Superovulation-Induced Mice

Superovulation treatment using PMSG and hCG induced the increase of E2 concentrations to double two days after PMSG and to ten times of their original values after another four days had elapsed, and stimulated the increase of P4 values as early as along with induced oestrus and the maximum levels of progesterone to around 100 ng/ml following superovulation in prepuberal cattle (Schneider *et al.*, 1980). The higher E2 and P4 levels and the unbalanced E2/P4 ratio may adversely affect estrus expression in the superovulated animal.

There is controversy about the effect of GH on estrus expression and E2 and P4 synthesis *in vivo and in vitro*. GH may stimulate the estrus expression by inducing the steroidogenesis in granulosa cells (Carlsson *et al.*, 1992; Harvey and Hull, 2001; Hsu and Hammond, 1987). Rieger *et al.* (1991) reported that co-treatment with rBST enhanced the superovulatory response in heifers by significantly increasing plasma progesterone concentrations at Day 6. However, GH may not affect steroidogenesis and estrus expression. Tapanainen *et al.* (1992) reported that GH administration significantly reduced serum E2 and P4 concentrations on the day of hCG injection and 1 day thereafter, did not affect the concentrations of E2 and P in the follicular fluid, but enhanced aromatase and 3-hydroxysteroid dehydrogenase mRNA in granulosa cells isolated from patients. Folch *et al.* (2001) insisted that the GH treatment did not significantly affect the percentage of ewes in estrus and the time of estrus onset.

This controversy about the effect of GH on E2 and P4 synthesis may be partly related to research conditions. Wathes *et al.* (1995) reported that GH alone (10 ng/ml to 10 μg/ml) did not stimulate either progesterone or IGF-I output and GH caused a marked increase of P4 in the presence of insulin. Sirotkin and Makarevich (1999) insisted that low concentrations (1~100 ng/ml) of GH stimulated, whilst a higher concentration (10 μg/ml) inhibited estradiol output, and low concentration of GH (1~100 ng/ml) slightly inhibited progesterone secretion in bovine ovarian granulosa cells. Considering Wilson's paper that GH levels were modestly increased during the follicular phase of the normal menstrual cycle, but the levels were markedly increased during superovulation with hMG or hMG plus GnRH agonists (Wilson *et al.*, 1991), Therefore, the superovulation treatment with gonadotropins may increase GH concentration and the increased GH may inhibit E2 and stimulate P4 synthesis in superovulated animal.

This study showed the results that GH should induce the estrus expression in superovulation-induced mice where the E2/P4 ratio was reduced by increasing less E2 level and more P4 level 12 hours before hCG injection, at peri-estrus stage. Nakamura *et al.* (2011) reported that GH should act to modulate estrogen and progesterone production differentially through endogenous IGF-I activity in granulosa cells, in which GH-IGF-I interaction leads to antagonization of BMP actions including suppression of FSH-induced progesterone production. Therefore, GH may increase the estrus expression by overcoming the superovulation problem of high E2/P4 ratio at peri-estrus stage.

GH and Pregnancy in Superovulation-Induced Mice

Pregnancy is closely related to ovulation, fertilization, embryo development and implantation.

High GH may stimulate ovulation in normal and transgenic mice. Cecim *et al.* (1995) reported that GH treatment in normal mice increased ovulation rate and high plasma bGH levels in transgenic females induced also higher ovulation rate. Danilovich *et al.* (2000) indicated that GH overexpression in transgenic mice significantly decreased the follicle apoptosis and increased the propensity for ovulation.

GH addition may stimulate fertilization *in vivo and in vitro*. The addition of eGH to *in vitro* maturation

medium influenced the *in vitro* nuclear and cytoplasmic maturation of equine oocytes (Pereira *et al.*, 2011), and *in vivo* administration of GH enhanced *in vitro* maturation and fertilization of human GV oocytes retrieved from small antral follicles (Hassan *et al.*, 2001). Folch *et al.* (2001) reported that the GH treatment significantly reduced the proportion of fertilized-ova and the number of accessory sperm attached to fertilize ova and the proportions of degenerate embryos recovered by reduced.

GH may stimulate embryo development *in vitro*. Montag *et al.* (2000) insisted that there was a highly significant increase in the rate of blastocyst formation and hatching when the embryos were exposed to GH *in vitro*.

GH may stimulate pregnancy when given to the recipient (Thatcher *et al.*, 2001). Kolibianakis *et al.* (2009) suggested that GH addition may increase the probability of clinical pregnancy and live birth in poor responders undergoing ovarian stimulation with GnRH analogues and gonadotrophins for IVF through meta-analysis. Yovich *et al.* (2010) reported that GH co-treatment significantly improved the clinical pregnancy rate by improving the oocyte and subsequent embryo quality.

The pregnancy is closely related to the E2 and P4 levels and the E2/P4 ratio at peri-ovulation and peri-implantation stages.

At peri-ovualtion stage that is related to increase of ovarian P4 level induced by preovulatory LH surge for ovulation, GH may affect E2 and P4 levels. GH increased progesterone synthesis by granulosa cells (Apa *et al.*, 1994) and in the presence of insulin (Wathes *et al.*, 1995). Thatcher *et al.* (2001) reported that sub-luteal plasma progesterone concentrations after insemination have been associated with lower pregnancy rates.

At peri-implantation stage that is characterized by high P4 level and low E2/P4 ratio in blood, GH may affect E2 and P4 levels. Especially, P4 facilitates implantation by stimulating the synthesis of enzymes responsible for lysis of the zona pellucida and maintain implantation through effects on both the maternal uterus and on the developing blastocyst (Graham and Clarke, 1997).

Many studies mentioned above suggested that GH stimulated ovulation, fertilization and implantation, resulting in higher pregnancy rate by increasing serum P4 level at peri-ovulation and peri-implantation stages. Also, this study showed the results that GH increased the pregnancy rate by decreasing E2/P4 ratio at peri-ovulation stage, but did not affect E2/P4 ratio at peri-implantation stage in superovulation-induced mice. Therefore, GH may induce high pregnancy rate by overcoming the superovulation problem of low P4 level and high E2/P4 ratio at peri-ovulation stage and peri-implantation stage.

GH and Fetus Number in Superovulation-Induced Mice

GH co-treatment enhanced the superovulatory response by improving the number of embryos in heifers (Rieger *et al.* 1991). Koch *et al.* (2010) reported that lambs born to ewes treated with a single injection of GH were larger at birth and had altered organ development and suggested that early maternal GH treatment may lead to permanent changes in the developing fetus.

This study showed the result that GH may stimulate gestation by increasing total fetus number in superovulated-mice.

In conclusion, GH could increase number of fetuses in superovulated-mice, through overcoming the superovulation problem, the higher E2 and P4 levels in the superovulated animal, that may adversely affect estrus, ovulation, fertilization, cleavage and implantation of embryos. That is, GH co-injected with PMSG should increase P4 level at peri-estrus stage in the superovulation-induced animal.

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