Superovulation following follicular synchronization with GnRH at random stages of the oestrous cycle in heifers: oocyte competence and *in vitro* embryo production

H. Kohram[1,2], V. Vahedi[1], A. Farahavar[1]

[1]Department of Animal Science, Faculty of Agriculture and Natural Resources, University of Tehran, Karaj, Iran
[2]Department of Clinical Sciences, Faculty of veterinary Medicine, Shahid Chamsan University, Ahvaz, Iran

ABSTRACT: The objective of this study was to develop a superovulatory program based on the synchronization of follicular waves with GnRH which could be applied regardless of the stage of the oestrous cycle. In this experiment, GnRH was given to 30 heifers in lactation between Days 0 and 7 (n = 13), 8 and 12 (n = 12), 13 and 16 (n = 5) of the oestrous cycle. Twenty-four heifers were used as controls and did not receive any GnRH. All follicles ≥ 6 mm were punctured 4 days after GnRH treatment in treated animals and between Days 8 and 12 of the oestrous cycle in control heifers. Two days after the follicular puncture, all heifers were superstimulated with 160 mg Folltropin-V given twice daily over 2 days. Oocytes were collected 42 h after the last FSH treatment. The oocytes were subjected to IVM/IVF and the developmental competence of embryos was compared. *In vitro* production of embryos was affected only by the stages of the oestrous cycle when the GnRH treatment was given and not by the GnRH treatment. No difference (P > 0.1) in the mean number of oocytes, cleavage and embryo production was noted between the control animals and the animals treated with GnRH in the late phase of the oestrous cycle. The mean number of blastocysts was higher (P < 0.05) in heifers treated with GnRH in the mid and the late phase of the oestrous cycle than in the early phase. In conclusion, the *in vitro* production of embryos was compromised in the present study with heifers following the follicular synchronization with GnRH. This procedure is advantageous for the *in vitro* production of bovine embryos since the spontaneous oestrus is eliminated. However, more investigations are needed to increase the competence of oocytes obtained following this procedure.

Keywords: heifer; oocyte; GnRH; oocyte competence

It is well accepted that the dominant follicle in the growing phase limits (directly or indirectly) the growth of the other follicles in the same wave (Guilbault et al., 1993). It has been shown that the injection of a GnRH agonist induces the emergence of a new follicular wave within 3 to 4 days after treatment and eliminates the large follicles by ovulation or luteinization (Thatcher et al., 1989; Twagiramungu et al., 1994; Pursley et al., 1995). In addition, the puncture of medium and large follicles in heifers at random stages of the oestrous cycle results in an FSH surge the next day and synchronous emergence of a new follicular wave 2 days after ablation (Bergfelt et al., 1994; Kohram et al., 1998; Cavalieri et al., 2001). The initiation of a follicular wave using these treatments allows the start of superstimulation at a random stage of the oestrous cycle (Kohram et al., 1998; Andrade et al., 2002).

GnRH treatment and follicle ablation at any stage of the oestrous cycle offer the advantage of initiat-

ing superstimulatory treatment immediately and ensuring that the treatment coincides with the time of the follicular wave emergence so that an optimal superovulatory response can be achieved. Furthermore, the entire oestrous cycle is available for superstimulation, eliminating the need for the oestrus detection and waiting 8 to 12 days before initiating the gonadotropin treatment (Bergfelt et al., 1994).

The number of collected transferable embryos did not increase by GnRH treatment and follicular aspiration 2 days prior to superstimulation, although the ovulatory responses increased by these treatments (Kohram et al., 1998). The effect of follicular synchronization with GnRH on a hormonal and follicular response prior to and during superovulation has been characterized (Kohram et al., 1998) but its effect on embryo production has not been defined clearly yet.

The objective of this study was to evaluate the effect of the GnRH-puncture protocol at any stage of the oestrous cycle prior to superstimulation on oocyte competence and on the *in vitro* production of bovine embryos.

MATERIAL AND METHODS

Animals and treatment

Fifty-four heifers were used in this study to determine if the treatment with GnRH given at any stage of the oestrous cycle before superstimulation influences the developmental competence of oocytes following *in vitro* fertilization (IVF) after FSH treatment. GnRH (100 μg Cystorelin; Sanofi, Quebec, Canada, im) was given to Holstein heifers (n = 30; 24 to 36 months of age) between Days 0 and 7 (Group 1, n = 13), 8 and 12 (Group 2, n = 12), 13 and 16 (Group 3, n = 5) of the oestrous cycle (oestrus = Day 0). Twenty-four heifers were used as control and did not receive any GnRH. All follicles ≥ 6 mm were punctured as previously described (Kohram et al., 1998) 4 days after GnRH treatment in treated animals and between Days 8 and 12 of the oestrous cycle in control untreated animals. Two days after the follicular puncture, all heifers were superstimulated with 160 mg Folltropin-V given twice daily over 2 days. All visible follicles (≥ 3 mm) were punctured 42 h after the last FSH treatment and immature oocytes were collected by transvaginal ultrasound guided follicular aspiration according to the method previously described (Pieterse et al., 1991). The oocytes collected from each heifer were subjected to a 24-h maturation period in TCM199 with 10% FBS (foetal bovine serum) and hormones (5 μg/ml LH, 0.5 μg/ml FSH, 1 μg/ml E2) at 39°C and 5% CO2 in humidified air. Matured oocytes were fertilized (Day 0) with frozen-thawed semen as previously described (Parrish et al., 1988). Cumulus cells were partially removed 18 h post fertilization and presumptive zygotes were co-cultured with oviductal epithelial cells in Menezo B2 medium supplemented with 10% oestrous cow serum. The cleavage rate was determined 48 h post fertilization and embryos were evaluated and classified on Day 7 of culture Grade 1 (excellent or very good), Grade 2 (good) and Grade 3 (fair), degenerated and unfertilized ova (IETS, 1990). Numbers of Grade 1 and 2, and of transferable embryos (Grade 1, 2 and 3) were also determined.

Statistical analysis

The mean numbers of oocytes or embryos produced (i.e. oocytes recovered and cultured as well as cleavage, blastocysts, quality 1 blastocysts) in GnRH-treated (n = 30) and untreated control (n = 24) heifers were analyzed by one-way analysis of variance using the GLM procedure of SAS. Data on the least-squares means between control and GnRH-treated animals (Group 1, n = 13; Group 2, n = 12; Group 3, n = 5) were compared using Duncan's multiple-range test.

RESULTS

The mean number of oocytes recovered and cultured did not differ (Table 1; $P > 0.1$) between control and GnRH-treated animals. The developmental competence of embryos obtained after IVM-IVF was compared between control and GnRH-treated heifers. A higher ($P < 0.05$) number of oocytes cleaved to the 2 cell embryo in control than in GnRH-treated animals. While no difference in the mean number of blastocyst production was noted between control and GnRH-treated heifers. Cleavage rate and blastocyst production were affected by the stages of the oestrous cycle when the GnRH treatment was given. In fact, the mean numbers of cleaved embryos and of the quality 1 embry-

Table 1. The mean number of recovered, cultured and cleaved oocytes and the blastocyst production

	Control	Total treatment	Treatment		
	n = 24	n = 30	Group 1, n = 13	Group 2, n = 12	Group 3, n = 5
Oocytes recovered (n)	10.6 ± 1.3 (255)	8.4 ± 1.2 (251)	8.0 ± 1.7 (104)	7.9 ± 1.7 (95)	10.4 ± 2.7 (52)
Oocytes cultured (n, %)	9.5 ± 1.2 (229, 90%)	8.0 ± 1.1 (240, 95%)	7.4 ± 1.7 (96, 92%)	7.9 ± 1.7 (95, 100%)	9.8 ± 2.7 (49, 94%)
Cleavage (n, %)	8.1 ± 0.8^{ac} (194, 85%)	5.1 ± 0.7^{b} (152, 63%)	4.4 ± 0.8^{d} (57, 59%)	4.9 ± 0.9^{d} (59, 62%)	7.2 ± 1.3^{c} (36, 73%)
Blastocysts (n, %)	4.2 ± 0.7^{c} (101, 44%)	3.0 ± 0.6 (90, 38%)	2.0 ± 0.6^{d} (26, 27%)	3.5 ± 0.6^{c} (42, 44%)	4.4 ± 0.9^{c} (22, 45%)
Quality 1 blastocysts (n, %)	2.9 ± 0.5^{ac} (71, 31%)	1.9 ± 0.5^{b} (56, 23%)	1.1 ± 0.5^{d} (14, 15%)	1.9 ± 0.6^{d} (23, 24%)	3.8 ± 0.9^{c} (19, 39%)

GnRH was given between Days 0 and 7 (Group 1; n = 13), 8 and 12 (Group 2; n = 12), or 13 and 16 (Group 3; n = 5) of the oestrous cycle (oestrus = Day 0); control untreated heifers (n = 24) did not receive any GnRH; all follicles ≥ 6 mm were punctured 4 days after GnRH treatment in treated animals and between Days 8 and 12 of the oestrous cycle in control untreated animals; all heifers were superstimulated with FSH 2 days after the follicular puncture

[a,b]in the same row indicate a significant difference between control and total treatment ($P < 0.05$)

[c,d]in the same row indicate a significant difference between control and treatment groups ($P < 0.05$)

os were higher ($P < 0.05$) when GnRH was given in the late stage of the oestrous cycle. The blastocyst production also was higher ($P < 0.05$) when GnRH was injected in the late stages (Groups 2 and 3) of the oestrous cycle.

DISCUSSION

Treatment with GnRH was used in the present experiment to synchronize follicular waves at any stage of the oestrous cycle and to expose a dominant follicle to puncture at a predictable time. As shown in a previous study (Kohram et al., 1998), this approach led to ovaries with a small number of large follicles ≥ 7 mm but with a large number of recruitable follicles 4 to 6 mm at the time of superstimulation initiation. Such ovarian conditions are similar to those observed in the absence of follicular dominance and have been shown to favour superovulatory responses (Guilbault et al., 1991; Huhtinen et al., 1992; Bungartz et al., 1994; Lucy, 2007). Dynamics of follicular and hormonal changes following the treatment with GnRH with or without follicular puncture has been reported (Kohram et al., 1998).

Since the animals were at different stages of the oestrous cycle, the population of follicles differed widely among stage-groups when GnRH was given. In response to GnRH, large follicles ovulate or become atretic depending on the stage of the oestrous cycle (Twagiramungu et al., 1995; Schneider et al., 2007) and as a result of the disappearance of large follicles, a new follicular wave emerges (Twagiramungu et al., 1995; Kohram et al., 1998; Martýnez et al., 2000; Garcia et al., 2004; Sato et al., 2005).

In this study the effect of follicular synchronization with GnRH on oocyte competence was investigated as the follicular environment may affect oocyte maturation (Ahmad et al., 1995; Hagemann et al., 1999; De Wit et al., 2000; Endriksen et al., 2000; Sirard et al., 2006). Results of this study indicated that the oocyte competence was affected by follicular synchronization with GnRH followed by the follicular puncture since the number of cleaved embryos was lower in GnRH treated animals than in the control. However, this decrease in treated animals was not observed in heifers treated with GnRH in the late phase of the oestrous cycle. A decrease in the number of cleaved embryos and blastocyst production in heifers treated with GnRH in the early phase of the oestrous cycle may suggest that the oocyte competence is reduced when GnRH was administered in the early phase of the oestrous cycle. The progesterone concentrations are low (Kohram et al., 1998) when GnRH treatment was

performed in the late stage of the oestrous cycle and this may suggest that the oocytes of follicles which develop in the presence of low concentrations of progesterone similar to the follicular phase of the oestrous cycle may have a high competence following *in vitro* fertilization. Moreover, preliminary results (data not shown) using a similar procedure, but in which luteolysis was induced during FSH treatment to reduce progesterone concentrations showed that follicles developing under such conditions yielded oocytes that had a greater competence for development into blastocysts (Jaiswal et al., 2006; Adams et al., 2008).

In summary, the previous study (Kohram et al., 1998) indicated that regardless of the stage of the oestrous cycle, the homogeneity of follicular inventories following the follicular synchronization is obtained within 4 days after GnRH treatment for follicles ≥ 7 mm but that follicular puncture is further needed to reach homogeneity within the population of recruitable follicles 4 to 6 mm before the initiation of superstimulation treatment. Although apparently normal in a preceding study with cows (Kohram et al., 1998), the *in vitro* production of embryos was compromised in the present study with heifers following the follicular synchronization with GnRH. This procedure is advantageous for the *in vivo* and *in vitro* production of bovine embryos since the spontaneous oestrus is eliminated, the procedure could be initiated at an unknown stage of the oestrous cycle without need for detection of the ovarian status at the initiation of superstimulation, and high follicular and ovulatory responses are obtained. However, more investigations are needed to increase the competence of oocytes obtained following this procedure.

REFERENCES

Adams G.P., Jaiswal R., Singh J., Malhi P. (2008): Progress in understanding ovarian follicular dynamics in cattle. Theriogenology, 69, 72–80.

Ahmad N., Schrick F.N., Butcher R.L., Inskeep E.K. (1995): Effect of persistent follicles on early embryonic losses in beef cows. Biology of Reproduction, 52, 1129–1135.

Andrade J.C., Oliveira M.A., Lima P.F., Santos Filho A. S., Pina V.M. (2002): Use of steroid hormone treatments prior to superovulation in Nelore donors. Animal Reproduction Science, 69, 9–14.

Bergfelt D.R, Lightfoot K.C., Adams G.P. (1994): Ovarian synchronization following ultrasound guided transvaginal follicle ablation in heifers. Theriogenology, 42, 895–905.

Bungartz L., Niemann H. (1994): Assessment of the presence of a dominant follicle and selection of dairy cows suitable for superovulation by a single ultrasound examination. Journal of Reproduction and Fertility, 101–3, 583–591.

Cavalieri J., Farin P.W., Kinder J.E., Van Camp S.D., Whitacre M.D., Waashburn S.P., Britt J.H. (2001): Ovarian follicular development following administration of progesterone or aspiration of ovarian follicle in Holstein cows. Theriogenology, 55, 805–821.

De Wit A.A., Wurth Y.A., Kruip T.A. (2000): Effect of ovarian phase and follicle quality on morphology and developmental capacity of the bovine cumulus-oocyte complex. Journal of Animal Science, 78, 1277–1283.

Endriksen P.J., Vos P.L., Steenweg W.N.M., Bevers M.M., Dielemans S.J. (2000): Bovine follicular development and its effect on the *in vitro* competence of oocytes. Theriogenology, 53, 11–20.

Garcia F.E.O., Cordero M.J.L., Hizarza E.A., Peralta O. J.G., Ortega C.M.E., Cardenas M., Gutierrez C.G., Sanchez T.E.M.T. (2004): Induction of a new follicular wave in Holstein heifers synchronized with norgestomet. Animal Reproduction Science, 80, 47–57.

Guilbault L.A., Grasso F., Lussier J.G., Rouillier P., Matton P. (1991): Decreased superovulatory responses in heifers superovulated in the presence of a dominant follicle. Journal of Reproduction and Fertility, 91, 81–89.

Guilbault L.A., Rouillier P., Matton P., Glencross R.G., Beard A.J., Knight P.G. (1993): Relationships between the level of atresia and inhibin content (alpha subunit and alpha-beta dimer) in morphologically dominant follicle during their growing and regressing phases of development in cattle. Biology of Reproduction, 48, 268–276.

Hagemann L.J., Beaumont S.E., Berg M., Donnison M.J., Ledgard A., Peterson A.J., Schurmann A., Tervit R.H. (1999): Development during single IVP of bovine oocytes from dissected follicles: interactive effects of estrous cycle stage, follicle size and atresia. Molecular Reproduction and Development, 53, 451–458.

Huhtinen M., Rainio V., Aalto J., Bredbacka P., Maki-Tanila A. (1992): Increased ovarian responses in the absence of a dominant follicle in superovulated cows. Theriogenology, 37, 457–463.

IETS (1990): Manual of the International Embryo Transfer Society, 2nd edition. Stringfellow D.A., Seidel S.M. (eds.): A Procedural Guide and General Information for the use of Embryo Transfer Technology, Emphasizing Sanitary Precautions. Champaign, Illinois, USA.

Jaiswal R.S., Singh J., Nagra H.S., Grafton T., Ratto M.H., Malhi P.S. (2006): Oocyte competence under different progestational environments. Biology of Reproduction, 109, 166 pp.

Kohram H., Bousquet D., Durocher J., Guilbault L.A. (1998): Alteration of follicular dynamics and superovulatory responses by gonadotropin releasing hormone and follicular puncture in cattle: a field trail. Theriogenology, 49, 1165–1174.

Kohram H., Twagiramungu H., Bousquet D., Durocher J., Guilbault L.A. (1998): Ovarian superstimulation after follicular wave synchronization with GnRH at two different stages of the estrous cycle in cattle. Theriogenology, 49, 1175–1186.

Lucy M.C. (2007): The bovine dominant ovarian follicle. Journal of Animal Science, 85, E89–E99.

Martýnez M.F., Adams G.P., Kastelic J.P., Bergfelt D.R., Mapletoft R.J. (2000): Induction of follicular wave emergence for estrus synchronization and artificial insemination in heifers. Theriogenology, 54, 757–769.

Parrish J.J., Susko-Parrish J., Winer M.A., First N.L. (1988): Capacitation of bovine sperm by heparin. Biology of Reproduction, 38, 1171–1180.

Pieterse M.C., Vos P.L.A.M., Kruip T.A.M., Wurth Y.A., Beneden T.H.V., Willemse A.H., Taverne M.A.M. (1991): Transvaginal ultrasound guided follicular aspiration of bovine oocytes. Theriogenology, 35, 857–862.

Pursley J.R., Mee M.O., Wiltbank M.C. (1995): Synchronization of ovulation in dairy cows using PGF2α and GnRH. Theriogenology, 44, 915–923.

Sato T., Nakada K., Uchiyama Y., Kimura Y., Fujiwara N., Sato Y., Umeda M., Furukawa T. (2005): The effect of pretreatment with different doses GnRH to synchronize follicular wave on superstimulation of follicular growth in dairy cattle. Journal of Reproduction and Development, 51, 573–578.

Schneider F., Tomek W., Grundker C. (2006): Gonadotropin-releasing hormone (GnRH) and its natural analogues: A review. Theriogenology, 66, 691–709.

Sirard M.A., Richard F., Blondin P., Robert C. (2006): Contribution of the oocyte to embryo quality. Theriogenology, 65, 126–136.

Thatcher W.W., Macmillan K.L., Hansen P.J., Drost M. (1989): Concepts for regulation of corpus luteum function by the conceptus and ovarian follicles to improve fertility. Theriogenology, 31, 149–164.

Twagiramungu H., Guilbault L.A., Proulx J., Ramkumar R., Dufour J.J. (1994): Histological populations and atresia of ovarian follicles in postpartum cattle treated with an agonist of gonadotropin releasing hormone. Journal of Animal Science, 72, 192–200.

Twagiramungu H., Guilbault L.A., Dufour J.J. (1995): Synchronization of ovarian follicular waves with a gonadotropin-releasing hormone agonist to increase the precision of estrus in cattle: a review. Journal of Animal Science, 73, 3141–3150.

Received: 2009–08–14
Accepted after corrections: 2009–05–11

Corresponding Author

Dr. Hamid Kohram, University of Tehran, Faculty of Agriculture and Natural Resources,
Department of Animal Science, Karaj, Iran
Tel. +98 261 224 8082, fax +98 261 224 6752, e-mail: kohram@can.ut.ac.in