Effects of re-immunization of heifers against inhibin on hormonal profiles and ovulation rate

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Abstract

To study the effect of re-immunization against inhibin on ovarian response and hormonal profiles, Japanese beef heifers ($n = 5$) were re-immunized three times with inhibin vaccine (recombinant ovine inhibin α -subunit in oil emulsion, $125 \mu\text{g ml}^{-1}$) one year after the primary immunization. Control heifers ($n = 5$) were injected with placebo (Montanide: Marcol adjuvant alone). Oestrous cycles were synchronized by using prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) and ovarian response was monitored daily by ultrasonography. Blood samples were collected by jugular venipuncture for assessment of hormonal levels and inhibin antibody titres. In contrast to controls, inhibin re-immunized heifers generated antibodies against inhibin rapidly reaching a peak level 9 days after the first booster injection. The mean concentrations of FSH in re-immunized cows increased significantly in comparison with controls. In addition, there was a significant increase in oestradiol- 17β and progesterone levels in re-immunized cows compared with controls. Inhibin re-immunized heifers had a significant increase in small ($\geq 4 < 7 \text{ mm}$), medium ($\geq 7 < 10 \text{ mm}$) and large ($\geq 10 \text{ mm}$ in diameter) sized follicles. Moreover, the mean ovulation rate was 5.0 ± 1.1 after the third booster injection in re-immunized heifers compared with control heifers (single ovulation). These results clearly demonstrate that re-immunization of inhibin can be used to enhance ovarian follicular development and ovulation rate. Furthermore, the great number of follicles is a potential source of oocytes that could be harvested for *in vitro* fertilization and embryo transfer programmes.

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Introduction

Previous studies support the importance of inhibins in the regulation of pituitary secretions of follicle-stimulating hormone (FSH) and negative correlation between FSH and inhibins has been reported (Beard *et al.* 1990, Kaneko *et al.* 1993a,b, 1995b, Tilbrook *et al.* 1993, Kishi *et al.* 1997, Ramaswamy *et al.* 1998, Araki *et al.* 2000). In domestic animals, induction of multiple ovulations is possible by administering exogenous gonadotrophins or by removing the inhibitory action of ovarian hormones on endogenous gonadotrophin release by the hypothalamus-pituitary axis. The use of a combination of equine chorionic gonadotrophin (eCG) and human chorionic gonadotrophin (hCG) has been widely used to induce superovulation. However, repeated eCG treatments lead to ovarian refractoriness and have clear negative effects

on reproduction (Bavister *et al.* 1986, Swanson *et al.* 1995, Roy *et al.* 1999).

In trials to develop alternative methods for superovulation in domestic animals, passive (Campbell & Scaramuzzi 1995, Kaneko *et al.* 1995b, Kusina *et al.* 1995, Akagi *et al.* 1997, Nambo *et al.* 1998, Medan *et al.* 2003a) and active (Fray *et al.* 1994, Glencross *et al.* 1994, Akagi *et al.* 2002, Medan *et al.* 2003b) immunizations against inhibins were used.

Although it is well established that passive or active immunization against inhibin neutralized endogenous inhibins and increased ovulation rate, further studies are needed to confirm the effect of re-immunization against inhibin on hormonal profile and ovulation rate. Therefore, the objective of the present study was to determine the effect of re-immunization of heifers with inhibin vaccine, 1 year after primary immunization, on gonadotrophins,

oestradiol and progesterone secretions, the number of follicles and ovulation rate.

Materials and Methods

Experimental design

This study was carried out on ten Japanese beef heifers weighing 455–510 kg, their ages ranged from 5 to 9 years. One year earlier, 5 heifers had been actively immunized against inhibin with a primary and two booster injections (immunized group) and the remaining 5 heifers were injected with a placebo and served as controls (control group). Oestrous cycles were synchronized by i.m. injection of 0.5 mg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) analogue (Estrumate, Sumitomo Pharm., Osaka, Japan). Thereafter, all heifers received a single i.m. injection of $PGF_{2\alpha}$ on day 18 of each oestrous cycle during the experimental period. On day 9 of the second oestrous cycle (day 0 = day of oestrus), the immunized group was injected intramuscularly with inhibin vaccine (recombinant ovine inhibin α -subunit in oil emulsion, $125 \mu\text{g ml}^{-1}$) followed by 2 boosters injected on day 9 of the third and fourth oestrous cycles. The control group was injected with a placebo (Montanide 888 (SEPPIC, Paris, France): Marcol 52 (EPPIC, Sydney, Australia) adjuvant alone). During each oestrous cycle, blood samples were collected every 12 h starting 9 days after oestrus until the end of oestrous cycle for hormonal assay and on day 9 and day 18 for determination of inhibin antibody titres. Blood samples were collected by jugular venipuncture into heparinized tubes, plasma was separated and stored at -40°C until assayed for hormones and inhibin antibody titres. Schematic representation of this protocol is shown in Fig. 1.

Preparation of inhibin vaccine

The α -subunit of ovine inhibin produced in *E. coli* by the recombinant DNA method (Forage *et al.* 1987) was used as an immunogen. The immunization dose was 1 ml of the immunogen ($125 \mu\text{g ml}^{-1}$) in Montanide 888:Marcol 52 (1:9) each time.

Figure 1 Schematic representation of the protocol used for re-immunization of heifers against inhibin. White arrows indicate time of $PGF_{2\alpha}$ injections, black rectangles indicate daily ultrasound examination and blood sampling every 12 h from day 9 to the end of each oestrous cycle for hormonal assay and • indicates a single blood sample on days 9 and 18 of each cycle for inhibin antibody titres.

Determination of the ovarian response

Ovarian follicular population and corpora lutea were determined daily from day 9 of each oestrous cycle until the end of oestrous cycle. Ultrasound scanner (SSD-650CL, Aloka, Tokyo, Japan) was used as described previously (Kaneko *et al.* 1991). Follicles were divided into three groups according to their diameter (small, $\geq 4 < 7$; medium, $\geq 7 < 10$ and large, ≥ 10 mm). In these Japanese beef cattle, follicles larger than 10 mm in diameter were considered to be preovulatory follicles in the normal oestrous cycle (Kaneko *et al.* 1995a). Ovulation rate was confirmed by counting the number of corpora lutea by ultrasonography between days 7 and 9 after oestrus.

Radioimmunoassays (RIAs)

Plasma concentrations of FSH were measured by RIA as described previously (Bolt & Rollins 1983) using anti-bovine FSH β subunit antiserum (USDA-5-pool), USDA-FSH-BP3 for radioiodination and US Department of Agriculture (USDA)-FSH-B1 as a reference standard. Plasma concentrations of luteinizing hormone (LH) were measured by RIA (Echternkamp *et al.* 1976) using anti-ovine LH serum (USDA-309-684P), USDA-bLH-I-1 for radioiodination and USDA-bLH-B-1 as a reference standard. The intra- and inter-assay coefficients of variation were 6.0% and 11.5% for LH and 3.0% and 9.4% for FSH, respectively.

Plasma concentrations of oestradiol- 17β and progesterone were determined by a double antibody RIA system using ^{125}I -labelled radioligands as described previously (Taya *et al.* 1985). Aliquots of 1 ml plasma for oestradiol- 17β and $100 \mu\text{l}$ for progesterone were extracted. Antisera against oestradiol- 17β (GDN 244) and progesterone (GDN 337) were provided by Dr G D Niswender (Animal Production and Biotechnology, Colorado state University, Fort Collins, CO, USA). In oestradiol- 17β assay, plasma samples were defatted with a mixture of 2 ml n-hexane and 0.5 ml acetonitrile to remove substances that could interfere with the estradiol- 17β assay as described by Nagata *et al.* (1996). The intra- and inter-assay coefficients of variation were 4.2% and 9.5% for oestradiol- 17β and 5.5% and 13.4% for progesterone, respectively.

Plasma inhibin antibody titres were determined as described by Kaneko *et al.* (1993a). Samples were diluted 1:12 with PBS containing 5% (w/v) bovine serum albumin and incubated for 24 h at 32°C with ^{125}I -labelled bovine 32 kDa inhibin ($5000 \text{ c.p.m. tube}^{-1}$) in a total volume of $300 \mu\text{l}$. Bound tracer was then separated by adding $100 \mu\text{l}$ PBS containing 1% (w/v) bovine γ -globulin and $500 \mu\text{l}$ PBS containing 25% (w/v) polyethylene glycol. The precipitate was counted following centrifugation at $1700 g$ for 30 min. Inhibin-binding capacity was expressed as a percentage of the total counts added.

Figure 2 Plasma levels of inhibin antibody titres (% binding of ^{125}I -labelled bovine inhibin at 1:12 dilution of plasma) in inhibin re-immunized ($\bullet$) and control ($\circ$) heifers measured at days 9 and 18 of each oestrous cycle. Values are mean $\pm$ S.E.M. ($n = 5$). Black arrows indicate time of immunization and white arrows indicate time of $\text{PGF}_{2\alpha}$ injection. $**P < 0.01$ compared with respective control value.

Statistical analysis

All values shown are mean $\pm$ S.E.M. ANOVA of repeated measures was used to examine the effect of inhibin immunizations on hormone levels and the number of follicles and corpora lutea. The significance of the difference between two means was determined by Student's t -test. A probability value (P) of less than 0.05 was considered to be significant. All statistical analyses were performed using the SAS computer package (SAS 1987).

Results

Inhibin antibody titres

As shown in Fig. 2, immunized animals showed a rapid increase in inhibin antibody titres, reaching a peak level 9 days after the first booster injection and remained at the peak level during the second and third injections of inhibin vaccine. On the other hand, the inhibin antibody titres in controls were consistently at nonspecific

Figure 3 Plasma concentrations of FSH (a) and LH (b) in inhibin re-immunized ($\bullet$; $n = 5$) and control ($\circ$; $n = 5$) heifers during 4 successive oestrous cycles. Black arrows indicate time of immunization and white arrows indicate time of $\text{PGF}_{2\alpha}$ injection. Values are mean $\pm$ S.E.M. Values under the horizontal bars are significantly different from corresponding values in control group. $*P < 0.05$.

binding values, as before the injection of placebo. Inhibin antibody titres were significantly higher compared with controls starting 9 days after the first booster injection until the end of the experiment.

Plasma concentrations of FSH and LH

Plasma concentrations of FSH and LH are shown in Fig. 3. There was no significant difference between the two groups before immunizations. About one week after first, second and third booster injections of inhibin vaccine the plasma concentrations of FSH showed a significant increase compared with controls. In contrast to FSH, plasma concentrations of LH did not differ significantly between inhibin-immunized and control groups.

Plasma concentrations of oestradiol-17 β and progesterone

Plasma concentrations of oestradiol-17 β rose sharply after injection of PGF_{2 α} . There was 3- or 4-fold increase in plasma concentrations of oestradiol-17 β after injection of inhibin vaccine compared with controls (Fig. 4a). Plasma concentrations of progesterone showed an abrupt decline after injection of PGF_{2 α} . In immunized heifers, there was a significant elevation in progesterone levels during

the third and 4th oestrous cycles compared with control heifers (Fig. 4b).

Ovarian activity and ovulation rate

After injection of PGF_{2 α} , all animals exhibited oestrus. The interval from PGF_{2 α} injection to the onset of pre-immunization oestrus was 85.8 ± 8.1 h and 78.4 ± 10.9 h in immunized and control groups, respectively. However, the overall mean interval from PGF_{2 α} injection to oestrus was shorter in the immunized group (58.3 ± 7.9 h) than in the control group (77.1 ± 5.5 h) during the three successive oestrous cycles following inhibin immunization. In addition, the numbers of follicles in immunized and control animals are shown in Fig. 5. In comparison with controls, inhibin immunized heifers had significantly more follicles. Small sized follicles increased earlier than the other categories after the first booster injection. All categories of follicles were significantly higher after the second and third booster injections of inhibin vaccine compared with controls. Ovulation rate during the four successive oestrous cycles is shown in Fig. 6. In contrast to pre-immunization, there was a significant increase in the ovulation rate in immunized heifers after the injection of first, second and third boosters of inhibin vaccine in comparison with control group (single ovulation).

Figure 4 Plasma concentrations of oestradiol-17 β (a) and progesterone (b) in inhibin re-immunized (●; $n = 5$) and control (○; $n = 5$) heifers during 4 successive oestrous cycles. Black arrows indicate time of immunization and white arrows indicate time of PGF_{2 α} injection. Values are mean $\pm$ S.E.M. * $P < 0.05$ compared with respective control value.

Figure 5 Number of (a) small follicles, $\geq 4 < 7$ mm in diameter; (b) medium follicles, $\geq 7 < 10$ mm in diameter; (c) large follicles, ≥ 10 mm in diameter and (d) total follicles in inhibin re-immunized ($\bullet$; $n = 5$) and control ($\circ$; $n = 5$) heifers during 4 successive oestrous cycles. Black arrows indicate time of immunization and white arrows indicate time of PGF_{2α} injection. Values are mean $\pm$ S.E.M.

Figure 6 The mean ovulation rate in inhibin re-immunized (solid bar; $n = 5$) and control (open bar; $n = 5$) heifers before and after injection of inhibin vaccine or placebo. Values are mean $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$ compared with control value.

Discussion

This study clearly demonstrated the efficiency of re-immunization against inhibin in increasing the number of follicles and ovulation rate in heifers, which is associated with elevated plasma concentrations of FSH. Re-immunization of Japanese beef heifers against inhibin one year after the first immunization rapidly stimulated an immune response and all immunized heifers generated antibodies that bound ^{125}I -labelled inhibin. Antibody binding was significant, confirming that increased ovulation rate after immunization with inhibin vaccine was due to immunoneutralization of endogenous inhibin. Immunoneutralization of endogenous inhibin diminished negative feedback on the anterior pituitary gland resulting in increased FSH secretion, subsequently increased follicular development and ovulation rate. There was a discrepancy in the previous studies about the effect of active immunization against inhibin on FSH secretions. Some reports (Findlay *et al.* 1989, Brown *et al.* 1990, Mizumachi *et al.* 1990, Wrathall *et al.* 1992, Medan *et al.* 2003b) demonstrated that active immunization against inhibin increased FSH secretions, meanwhile others (Schanbacher *et al.* 1991, Tannetta *et al.* 1998, Hennies *et al.* 2001) found an increase in ovulation rate without an increase in FSH levels. In the present study, we found that re-immunization against inhibin clearly increased FSH secretions. These findings suggest that inhibin may have local auto/paracrine effects within the ovary and neutralization of one of these effects may augment FSH in enhancing follicular growth. In sheep, active immunization against inhibin increased intrafollicular activin A, unaccompanied by a rise in the concentration of its binding protein (follistatin) which, in turn, might enhance follicular growth and ovulation rate (Tannetta *et al.* 1998). In contrast to the observed increase in FSH concentrations following re-immunization against inhibin, concentrations of LH did

not show a significant difference between inhibin vaccinated and control heifers in the present study.

The higher level of plasma oestradiol- 17β in immunized heifers in the present study is probably due to an increased number of oestrogenic follicles destined to ovulate. Similarly, goats actively immunized against inhibin showed a significant increase in circulating oestradiol concentrations (Hennies *et al.* 2001, Medan *et al.* 2003b). In addition, the elevated plasma progesterone levels in inhibin re-immunized heifers reflects the increased ovulation rate and increased number of corpora lutea.

Multiple ovulations have been induced successfully by passive and active immunization against endogenous inhibin in domestic animals (Wheaton *et al.* 1992, 1996, Konishi *et al.* 1996, Akagi *et al.* 1997, Takedomi *et al.* 1997, Nambo *et al.* 1998, Medan *et al.* 2003a,b) and laboratory animals (Rivier & Vale 1989, Kishi *et al.* 1996, Shi *et al.* 2000, Wang *et al.* 2001). In the present study, ovulation rate increased in re-immunized heifers after the first, second and third booster injections of inhibin vaccine, indicating that repeated injection of inhibin vaccine can be used for inducing superovulation without any adverse effect on ovulation rate. Moreover, the great number of medium and large sized follicles recorded in heifers injected with inhibin vaccine is a potential source for oocytes necessary for *in vitro* and embryo transfer programs. This may help in the production of cloned or transgenic cattle and the establishment of oocyte banks for superior breeds, especially with the availability of transvaginal ultrasound-guided follicular aspiration, which proved to be a non-stressful technique for repeated harvesting of oocytes from cows (Chastant-Millard *et al.* 2003).

In summary, re-immunization of cows with inhibin vaccine produced antibodies that neutralized endogenous inhibin and increased circulating FSH. In addition, the great number of follicles and increased ovulation rate indicate that inhibin immunization can be used repeatedly for induction of superovulation without any additional injections of exogenous gonadotrophins. Therefore, this study confirms that inhibin vaccine is a practical and repeatable method for promoting superovulation in heifers and that great number of follicles could be aspirated and used for *in vitro* fertilization and embryo transfer programs.

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