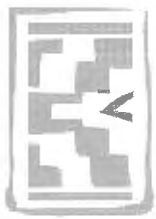

Fertility in rats immunized with steroid-free bovine follicular fluid

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ABSTRACT

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Inhibin is a gonadal hormone that inhibits the release of follicle stimulating hormone (FSH) from the anterior pituitary gland. The objective of this study was to determine whether active immunization of male and female rats against inhibin rich, steroid-free bovine follicular fluid would increase inhibin antibody titre, onset of female puberty, pregnancy rate, litter size, testis weights, testosterone concentration and serum FSH. Immunization of rats with steroid free bovine follicular fluid stimulated production of anti-inhibin antibodies that immunoneutralized endogenous inhibins and increased levels of circulating FSH in immunized males. Inhibin immunoneutralization resulted in early vaginal opening in immunized females compared with controls and pregnancy rates were increased when immunized female rats were mated with immunized males. However, serum testosterone, testis weights and potential litter size remained unchanged. We conclude that methods to immunoneutralize inhibin may have merit as therapeutic procedures to enhance reproductive performance in domestic animals.

Keywords: Fertility, FSH, immunoneutralization, inhibin

INTRODUCTION

Antibodies to steroid and peptide hormones are used extensively to study endocrine regulation of reproduction. In recent times they have been commercially exploited as therapeutic agents to manipulate reproduction (Scaramuzzi, Campbell & Martin 1993). An example of a hormone currently under investigation as a therapeutic agent to enhance fertility in domestic animals is inhibin.

Inhibin is a heterodimeric glycoprotein hormone ($\alpha\beta$) produced primarily by the gonads, that feeds back to the anterior pituitary gland to selectively inhibit synthesis and/or release of follicle stimulating hormone (FSH) (Burger & Igarashi 1988; Rivier, Schwall, Mason, Burton, Vaughan &Vale 1991). Inhibin is produced by granulosa cells of the ovary and by Sertoli cells of the testis. The richest source of inhibins is ovarian follicular fluid. In the female, FSH is responsible for follicular recruitment and thus determines the ovulation rate (McLeod & McNeilly 1987). In the male, FSH determines the rate of spermatogenesis, increases the mitotic rate of Sertoli cells, increases seminiferous tubule length and the relative mass of spermatocytes and spermatids in prepubertal animals (Swanlund, N'Diaye, Loseth, Pryor & Crabo 1995). Several investigators report that active immunization (Voglmayr, Mizumachi, Washington, Chen & Bardin 1990; Martin, Williams & Ireland 1991; Schanbacher 1991; Glencross, Bleach, Wood & Knight 1994; Moreau, Satterlee, Rejman, Cadd, Kousoulas & Fioretti 1998; Bame, Dalton, Degelos, Good, Ireland, Jimenez-Krassel, Sweeney, Saacke & Ireland 1999) or passive immunization (Kaneko, Nakanishi, Akagi, Arai, Taya, Watanabe, Sasamoto & Hasegawa 1995; Kusina, Meyer, Carlson & Wheaton 1995; Sewani, Bagdasarian, Ireland & Bagdasarian 1998) against inhibin leads to elevated levels of

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serum FSH. In most of these studies it is possible to relate the increase in FSH levels to increased ovulation rates or sperm production, thus providing a rationale to develop inhibin-based antigens as therapeutic fertility-enhancing vaccines for domestic animals. Although the aforementioned immunization studies consistently show improved rates of ovulation and spermatogenesis, there are few reports on the effect of inhibin immunoneutralization on pregnancy rates, embryo number or litter size, reproductive parameters that are of interest to the farmer.

In this study, male and female rats were actively immunized with steroid free bovine follicular fluid to investigate the effects of inhibin immunoneutralization on male and female fertility.

MATERIALS AND METHODS

Animals and treatments

Rats were purchased from and maintained by the University of Zimbabwe Animal House. Weaned male and female Sprague-Dawley rats (21 days old) were housed five to a cage and maintained on a natural night : day cycle, and provided with food and water *ad libitum.* Females were checked for vaginal opening on a daily basis.

Bovine follicular fluid

Since ovarian follicular fluid is the richest source of inhibins, we used bovine follicular fluid (bFF) in our study. Bovine ovaries were collected from non-pregnant cows at a local abattoir, and bFF was aspirated from ovarian follicles greater than 3 mm in diameter and pooled. After centrifugation (2 000 x *g*, 15 min, 4 °C) to remove cellular debris, bFF was incubated overnight at 4 °C with activated charcoal (25 mg/mℓ) to remove steroids. After another centrifugation step, the steroid free supernatant was further clarified through Sep-Pak C18 cartridges (Millipore, Milford, MA). This steroid free bFF (65 mg/mℓ protein) was used to immunize rats.

Immunization of rats

Steroid-free bFF was used to actively immunize male and female rats. Beginning at 21 days of age, 20 male and 20 female rats were injected subcutaneously (s.c.) over a 12-week period at 2-week intervals with 50 µg bFF protein mixed with Freund's adjuvant. Another 20 male and 20 female control rats received Freund's adjuvant only. Females were checked daily for vaginal opening which is the initial sign of onset of puberty. Two weeks after the final booster, at 4 months of age, blood samples were collected from all animals by heart puncture for determination of inhibin antibody titre and for hormone assays. Serum for hormone assays was stored at –20 °C until assayed. The following day, rats were caged in pairs (10 pairs per mating group) and mated for 5 days as follows:

- Group A – control males with control females
- Group B – control males with immunized females
- Group C – immunized males with control females
- Group D – immunized males with immunized females

After the 5-day period of co-habiting, males were separated from females. Fourteen days after first day of mating (potentially day 14 of pregnancy) the females were euthanased by chloroform inhalation. Pregnancy rates (percent females impregnated) and potential litter size (number of embryos in the uterus) were assessed in females. Males were euthanased on the same day and their testicular mass were recorded and their blood collected for assays. Antibody titres were determined the day after blood collection and hormone assays were performed four weeks after termination of the experiment.

Titre of anti-inhibin antibodies

Nunc Maxisorp microtiter plates (Nunc Inc., Denmark) were coated overnight at room temperature with 1 µg/well synthetic porcine inhibin a subunit, fragment 1-32 (pINHa$^{1-32}$; Sigma, St Louis, MO) dissolved in 10 mM phosphate buffered saline pH 7,4 (PBS). To block non-specific binding sites, each well was incubated for 1 h with 1 % bovine serum albumin (BSA, Sigma, St Louis, MO) dissolved in PBS containing 0,05 % Tween-20 (PBS-Tween). ELISA plates were then incubated with rat serum diluted 1: 5 000 in PBS-Tween containing 0,1 % BSA (PBS-Tween-BSA) for 2 h followed by 1 h incubation with horse radish peroxidase-labelled goat anti-rat IgG (Sigma, St Louis, MO) diluted 1:5 000 in PBS-Tween-BSA. Plates were washed four times with PBS-Tween between all incubation steps. Colour was developed by incubating plates with ortho-phenylenediamine (Sigma, St Louis, MO) diluted in 0,1 M citrate buffer, pH 4,5, containing 0,01 % H_2O_2. The titre of anti-inhibin antibodies = absorbance at 490 nm (A_{490}) read from a microplate reader.

Hormone assays

Luteinizing hormone (LH) and follicle stimulating hormone (FSH)

Because female oestrous cycles were no synchronized in this study, LH and FSH were measured in the serum of the male rats only, using a homologous radioimmunoassay (RIA). Rat FSH and LH reagents were kindly supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK).

A previously described RIA was used to determine concentrations of FSH or LH in serum in duplicate (Sewani *et al.* 1998). Samples were analyzed in a single assay for each hormone. The FSH and LH assay sensitivities were 0,53 and 0,26 ng/mℓ and intra-assay coefficients of variation (CV) were 2,3 and 4,6 % (n = 40), respectively. Cross-reaction of FSH with LH and of LH with FSH is < 2 % (per NIDDK guidelines).

Testosterone

Serum concentrations of testosterone were determined using the Coat-A-Count Total Testosterone assay kit from Diagnostic Products (DPC, Los Angeles, CA), per manufacturer's instruction for a non-extraction assay. Briefly, 50 µℓ of rat serum was incubated with 1 mℓ I^{125}-labelled testosterone tracer in antiserum-coated tubes at 37 °C for 3 h. Coated tubes were decanted, and radioactivity bound to the dried tubes determined in a Cobra II gamma counter (Packard). All samples were analyzed in a single assay. Assay sensitivity was 0,2 ng/dℓ and intraassay CV was 1,2 % (n = 40 samples). Cross reactivities are:

oestradiol	= 0,02 %
5α-dihydrotestosterone	= 3,4 %
other steroids	= < 1 % (per DPC guidelines)

Statistical analysis

All values are presented as means ± SEM. Testis mass, litter size, testosterone, FSH and LH levels were compared by unpaired student's t-test. Chi-squared analysis was used to compare the effect of immunization on pregnancy rates. Values were considered significant at $P < 0,05$.

RESULTS

Titre of inhibin antibodies

Immunized male and female rats had a greater ($P <$ 0,05) mean titre of anti-inhibin antibodies compared to controls. Mean titre of anti-inhibin antibodies was:

A_{490} = 0,1 ± 0,05 for controls

A_{490} = 0,765 ± 0,022 for immunized males

A_{490} = 0,839 ± 0,170 for immunized females

There was no significant difference between immunized male and female rat titre of anti-inhibin antibodies (Fig. 1).

Serum hormone concentrations

Male rats immunized against inhibin had a significantly higher (61 %; $P < 0,05$) serum concentration of FSH compared with controls. In contrast, serum LH and testosterone concentrations were similar for inhibin-immunized rats and controls (Fig. 2).

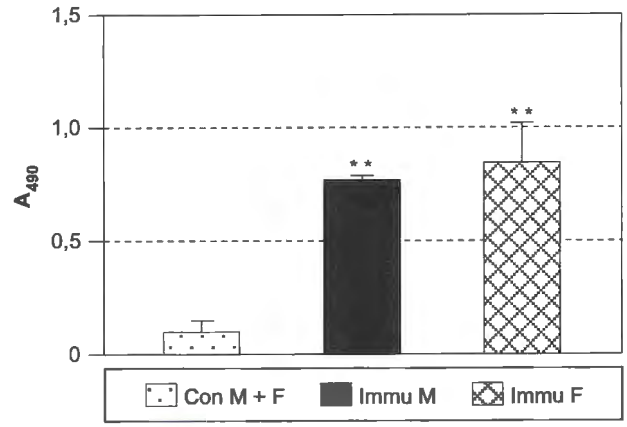

FIG. 1 Mean (± SEM) serum titre of anti-inhibin antibodies, given as absorbance at 490 nm (A_{490}) in an ELISA, for control (Con) and bFF immunized (Immu) male (M) and female (F) rats (n = 20).
** $P <$ 0,001 compared with controls

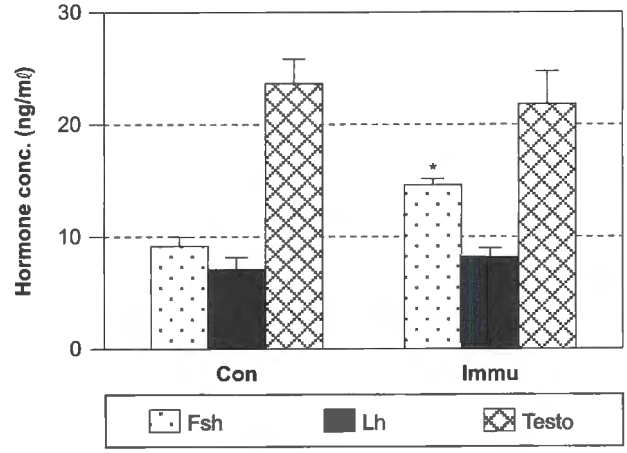

FIG. 2 Mean (± SEM) serum follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone (TESTO) in control (CON) and bFF immunized (IMMU) male rats
* $P <$ 0,05 compared to controls

Vaginal opening

Immunized females showed significantly earlier ($P <$ 0,05) opening of the vagina compared to controls. Vaginal opening occurred at 39,3 ± 0,4 days in controls compared to 36,4 ± 0,6 days in inhibin-immunized rats.

Pregnancy rates and potential litter size

Immunized females mated with immunized males showed higher ($P < 0,05$) pregnancy rates (100 %) compared with other mating groups (Fig. 3A). The

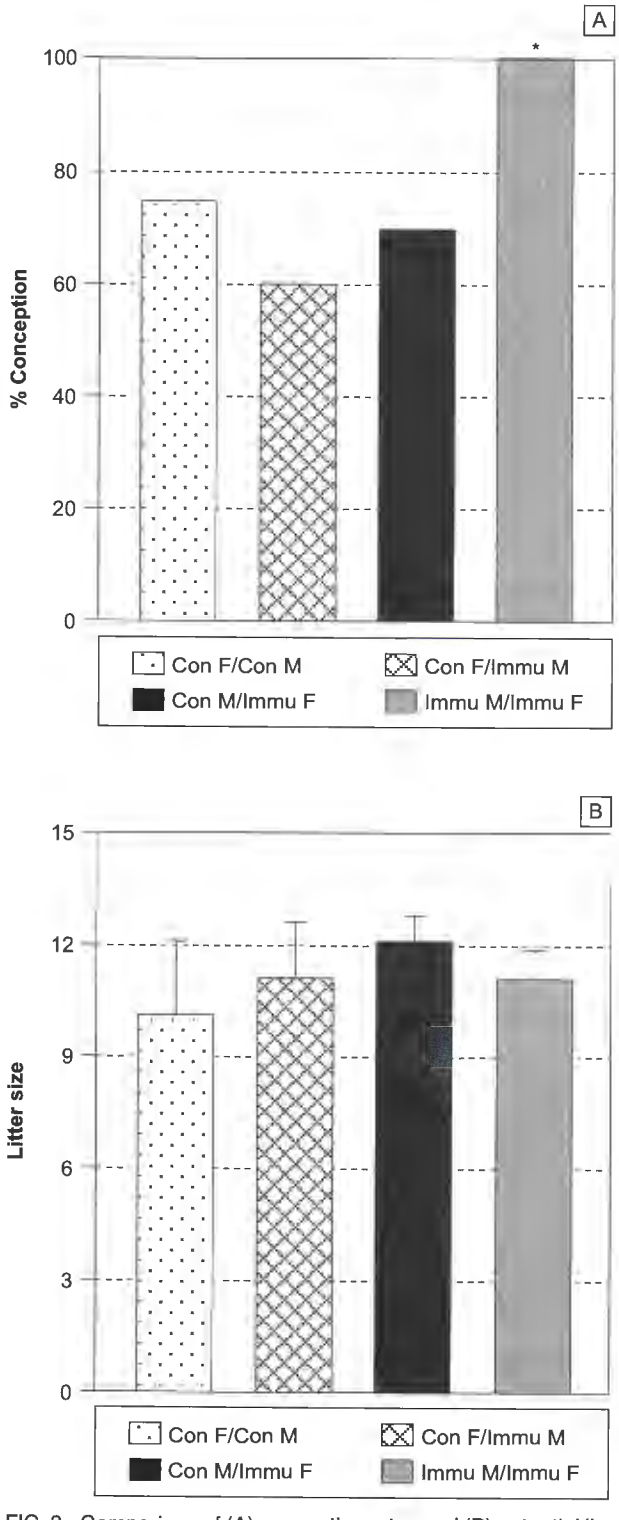

FIG. 3 Comparison of (A) conception rates and (B) potential litter size between various mating groups of control (Con) and bFF immunized (Immu) male (M) and female (F) rats

* $P < 0,05$ compared to controls

rest of the mating groups showed similar conception rates. There was no significant effect of immunization on litter size amongst all groups (Fig. 3B).

Testis mass (sperm production index)

There was no significant effect of inhibin immunization on mean testis mass for control (3,25 g ± 0,05) *vs* bFF (3,26 g ± 0,03) treated mice.

DISCUSSION

We have demonstrated that immunization of rats with steroid-free bovine follicular fluid mixed with Freund's adjuvant stimulated production of anti-inhibin antibodies that immunoneutralized endogenous inhibins and increased levels of circulating FSH in immunized males. Inhibin immunoneutralization resulted in early vaginal opening in immunized females compared with controls and pregnancy rates were increased when immunized female rats were mated with immunized males. However, serum testosterone, testis mass and potential litter size remained unchanged.

Immunization of rats with steroid free bFF stimulated production of anti-inhibin antibodies in both male and female rats. The anti-inhibin antibodies generated by immunization bound and immunoneutralized endogenous inhibins resulting in increased levels of FSH in males. Interestingly, groups of animals that showed the highest anti-inhibin antibody titre in an ELISA also showed the highest elevations in FSH, supporting previous observations in immunized adult rams (Voglmayr *et al.* 1990) and bulls (Bame *et al.* 1999). Furthermore, the enhanced serum concentrations of FSH observed in this study support previous findings in which serum concentrations of FSH are increased after active immunization of young bulls or adult rams against α-subunit of inhibin (Voglmayr *et al.* 1990; Martin *et al.* 1991; Schanbacher 1991; Bame *et al.* 1999).

Although FSH levels were elevated, there was no effect of immunization on testicular mass. During development, male rats show peripubertal increase in serum concentrations of FSH, LH and androgens starting from 20–50 days postpartum (Selmanoff, Goldman & Ginsgurg 1977). The elevation in FSH is responsible for the maximal growth spurt of testes, seminal vesicles, preputial and prostate glands, and also initiates spermatogenesis through the interaction with testosterone (Selmanoff *et al.* 1977). In support of this observation, administration of FSH increases the mitotic rate of Sertoli cells, seminiferous tubule length and the relative mass of spermatocytes and spermatids in prepubertal boars (Swanlund *et al.* 1995). These observations imply that in order to affect testicular mass, FSH levels should increase within the 20–50 day window. Our results imply that FSH elevation in male rats may have occurred after the critical testicular development window and thus, testicular mass were not affected. However, in domestic animals that have a long prepubertal period,

this FSH window is longer and thus testicular effects are observed (Voglmayr *et al.* 1990; Martin *et al.* 1991; Schanbacher 1991; Bame *et al.* 1999). There was no difference in testosterone levels between inhibin-immunized males and controls. Previous studies have shown no change (Martin *et al.* 1991), a decrease (Sewani *et al.* 1998) or increase (Bame *et al.* 1999) in testosterone levels after inhibin immunoneutralization. The reason for this variability in testosterone levels is not clear, but may be attributed to local testicular effects of inhibin immunoneutralization.

The early vaginal opening observed in female rats implies an accelerated onset of puberty in female rats. Vaginal opening is the first sign of pubertal onset followed 3 or 4 days later with oestrus cycling. Prolonged reproductive life span of the female is of interest because this may imply potentially increased productivity per female per lifetime. Previous studies in inhibin immunoneutralized ewes demonstrated an acceleration of puberty onset and in a separate study, accelerated return to oestrus of ewes after winter anoestrus (O'Shea, Bindon, Forage, Findlay & Tsonis 1993; McLeod, Hunter, Bleach, Glencross & Wrathall 1992). Thus, our present observation confirms previous findings that inhibin immunoneutralization stimulates early onset of puberty in female rats.

The improved pregnancy rates observed when immunized male and female rats were mated imply an improved reproductive performance in immunized animals. We were, however, surprised that mating immunized rats with unimmunized rats resulted in conception rates similar to controls. For improved conception rates to occur, both males and females have to be immunized against inhibin. Despite the improved conception rates, potential litter size was not affected by inhibin immunoneutralization. Litter size is determined by rate of ovulation, size of uterine space and availability of energy to support the litter. Any one of these factors may have affected litter size in the present study. However, since inhibin immunoneutralization results in increased ovulation rates and sperm output when FSH elevations occur at critical times in the development of animals, it is conceivable that in our study FSH elevations may have occurred too late to increase gametogenesis (McKeown, Callaghan Roche & Boland 1997; Bame *et al.* 1999). Furthermore, although the bFF used in this study was steroid free, it still contained other bioactive components whose effects may have contributed to the results we observed.

On the basis of our results, we conclude that inhibin immunoneutralization increases FSH release in males, stimulates sexual development in females and improves conception rates. Thus, methods to immunoneutralize inhibin may have merit as therapeutic procedures to enhance reproductive performance in domestic animals.

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