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Effects of Anti-inhibin Free α Immunization on Ovulation, *in Vitro* Fertilization, and Embryo Development in Mice

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ABSTRACT

Inhibin is a dimeric glycoprotein comprised of two subunits, α and β. Immunization against dimeric inhibin is mainly used in assisted reproductive technology to induce superovulation. However, the specific function of immunoreactive-free inhibin α subunit remains unclear. In this study, two main investigations were conducted (first on ovulation and the other on fertilization) using a novel monoclonal antibody targeting free inhibin α subunit (Pro-αN-αC). The ovulation study was conducted in 6 replicates, involving a total of 48 female CD1 mice aged 4–6 weeks. In each replicate, 4 control mice received PMSG/hCG treatment, and 4 treated mice received PMSG/hCG with mAb- Free α subunit. The fertilization study was conducted in 3 replicates, involving a total of 22 female CD1 mice. In each replicate, there were 4, 3, and 4 mice respectively for both control and treatment groups. In both investigations, female mice were injected intraperitoneally with 50 units/ml of Pregnant Mare Serum Gonadotropin (PMSG), alone or combined with 400ug of mAb- Free α subunit, followed by an injection of 50 units/ml of Human Chorionic Gonadotropin (hCG) 48 hours later. Seventeen hours post-injection, the females from all groups were sacrificed, and the ovulated oocytes were collected from the oviducts. For the fertilization study, *in vitro* fertilization was performed using fresh sperm from male CD1 mice. The results revealed that neutralization of the free inhibin α subunit significantly decreased the ovulation rate by 47.29% compared to the control group, while immunoneutralization significantly increased the fertilization rate by 55.68% and the blastocyst development by 43.85% compared to the control group. This study suggests that immunization against free inhibin α subunit decreases ovulation, in contrast to the effect of immunoneutralization of dimeric inhibin. The authors hypothesize that the free α subunit may function as an inhibin antagonist, competing with inhibin for binding to its co-receptor.

Keywords: Activin, Betaglycan, Fertilization, Immunoneutralization, Inhibin, Ovulation

INTRODUCTION

The use of genetically modified mice as models of human disease in life science research provides detailed insights into disease mechanisms and therapeutic strategies for rare diseases and pathological conditions (Kaushik et al., 2024; Zhong et al., 2024). To achieve efficient production and maintenance of genetically modified mice, various protocols based on superovulation are used (Guan et al., 2012). However, as the new transgenic technologies are improving, the number of mutant mice is increasing exponentially, and so is the number of female oocyte donors. The success of *in vitro* fertilization (IVF) is very much dependent on the quality of the oocyte/embryo and on a culture system that supports the development of healthy embryos capable of reaching their implantation potential (Sciorio et al., 2024). The production of large numbers of mature, high-quality oocytes is of great importance for assisted reproduction techniques (ARTs). Superovulation is a major component of embryo transfer and transgenic technologies; it facilitates the generation of genetically engineered mice/embryos and reduces the number of animals used. Superovulation techniques are based on the induction of follicle maturation and ovulation through the administration of hormones (Kaneko and Garrels, 2020).

Since the late 1980s, standard superovulation using a combination of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) has been introduced in the production of transgenic mice to induce follicular development and ovulation of a large number of oocytes from a limited number of female mice (Fleming and Yanagimachi, 1980). Ovulation rates have also been enhanced by vaccination against inhibin in many species including mice (Mochida, 2020; Hasegawa et al., 2022) rats (Mochida et al., 2024), guinea pigs (Shi et al., 2000), goats (Medan et al., 2003) and heifers (Bleach et al., 2001). Inhibin is a heterodimeric glycoprotein, mainly secreted by granulosa cells in

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response to follicle-stimulating hormone (FSH) release and to inhibit FSH secretion from the pituitary gland, thus regulating the number of developing follicles and ovulated oocytes (Makanji et al., 2014). The mature form of inhibin, with a molecular weight of 32-34 kDa, consists of an αC subunit (~20 kDa) disulfide-linked to either a βA or a βB subunit (~13 kDa), forming inhibin A or inhibin B, respectively (Vale et al., 1988; Makanji et al., 2014). However, immunoreactive free inhibin α subunits (~50-58 kDa) are also found in abundance, though their specific function remains unclear (Findlay et al., 2001; Laird et al., 2019). While some studies suggest that the free form of inhibin α subunit may have an antagonist effect on the mature form of inhibin (Drummond et al., 2004; Laird et al., 2019). there is no experimental evidence to support this hypothesis.

To date, no specific receptor has been identified for inhibin that associates with its signaling pathway (Robertson et al., 2000; Bernard et al., 2020). However, the physiological effects of inhibin are generated by a system of competition with activin receptors, leading to the inhibition of activin action (Bernard et al., 2020). Unlike inhibin, activins stimulate FSH secretion from the pituitary gland and are composed of two β-subunits, which are also shared with inhibin (Gray et al., 2005). Inhibin's antagonism of activin depends on interactions with betaglycan, a cell-surface proteoglycan co-receptor, also known as TGFBR3 (Lewis et al., 2000; Makanji et al., 2007). Betaglycan binds inhibin to form a stable complex with a high affinity for type II activin receptors, thereby antagonizing activin-mediated receptor activation (Lewis et al., 2000). In addition, a recent study indicates that inhibin B acts preferentially via another gonadotroph-specific co-receptor, called transforming growth factor-beta receptor 3 (TGFBR3L), to suppress FSH secretion (Brûlé et al., 2021).

A great majority of protocols used in assisted reproductive technology (ART) and superovulation are based on the immunoneutralization of endogenous inhibin (Mochida, 2020). However, given the limited understanding of the inhibin signaling pathway and the potential role of its immunoreactive-free α subunit, the success of superovulation protocols is not always guaranteed. In some studies, these protocols have surprisingly failed to induce ovulation (Drummond et al., 2004). In fact, evidence suggests that free α inhibin may have a biological activity distinct from that of inhibin in the female reproductive system (Mason et al., 1996; Drummond et al., 2004). Therefore, this study aimed to investigate the effects of immunoneutralizing the free inhibin α subunit (Pro-αN-αC) on mice ovulation, *in vitro* fertilization, and embryo development. The study employed a novel monoclonal antibody developed against 13-amino acid epitope; <^{64}HAVGGFMHRTSEPE>, within the mouse inhibin αN region. There is a lack of studies in this area to explore the physiological role of free inhibin α subunit and provide new insights to improve ART protocols and fertility treatments based on inhibin.

MATERIALS AND METHODS

Ethical approval

All experiments were approved by the University of California San Francisco guidelines under Institutional Animal Care and Use Program (IACUC) approved protocols (AN203067).

Animals

A total of 98 outbred CD1 mice strains (95 females and 3 males) were purchased from Charles River Laboratories, USA, for the entire study. The research was conducted at the University of California San Francisco, Cryopreservation Core. The female mice, used as oocyte donors, were 4-6 weeks old with an average weight of 18 grams, and the male mice, used as sperm donors, were 12 weeks old with an average weight of 40 grams. Throughout the study, the animals were housed under a monitored light/dark cycle, (light from 07:00 to 19:00), provided with water and fed *ad libitum*, and checked daily by the animal care staff.

Anti-Inhibin free α subunit monoclonal antibody

Bioinformatic analysis was initially conducted to select a suitable peptide antigen for antibody production. The sequence of mouse inhibin α (*Mus musculus*) was obtained from the NCBI database. The peptide was created through GenScript's Optimum Antigen Design Program, and was optimized using the industry's most advanced antigen design algorithm. The peptide was measured against several protein databases to confirm the desired epitope specificity. Benefits of using the Optimum Antigen Design Tool include avoidance of unexposed epitopes, ability to specify desired cross-reactivity, strong antigenicity of chosen peptide, identification of the best conjugation, and presentation options (GenScript, 2002). Using the GenScript Optimum Antigen design tool, the sequence with the highest immunogenicity within the Inhibin α Subunit Pro-αN was identified, specifically <HAVGGFMHRTSEPE>. The 13-amino-acid sequence '^{64}HAVGGFMHRTSEPE-C-KLH' was then chemically synthesized by GenScript (Lot No.: 95490490005/pe3623). To enhance its immunogenicity, the peptide was conjugated to keyhole limpet hemocyanin (KLH) via a cysteine residue. A

monoclonal antibody was then produced using hybridoma technology. following the protocol described by Yokoyama et al. (2013) and Yokoyama et al. (2013).

A group of 5 female Balb/c mice (6 to 8 weeks old) were intraperitoneally injected with 50 µg of the chemically synthesized peptide mixed with 1:1 Complete Freund's adjuvant (Sigma-Aldrich, Germany). A control group of 3 female Balb/c mice received normal saline only. After 15 days, a booster immunization was administered, consisting of 50 µg of antigen mixed with 1:1 Incomplete Freund's adjuvant Sigma-Aldrich, Germany) and injected intraperitoneally. Three days after the booster, the animals were prepared for cell fusion. Hybridoma cells were generated by fusing spleen cells from immunized female Balb/c 6-week-old mice with SP2O myeloma cells. The supernatant from these cultures was initially screened using a homemade ELISA test, developed using the synthesized peptide antigen, according to the protocol described by Holzlöhne et al. (2017) and Holzlöhner and Hanack (2017). Following this, a stable antibody-secreting cell line was expanded, and the monoclonal antibody was then purified using a Sephadex G-200 column. The monoclonal antibody was characterized as IgG Kappa and was shown to be immunospecific only to the Free Inhibin α Subunit Pro-αN-αC (~52 kDa) in Western blotting analysis (unpublished observations).

Reagents

All reagents used in this study were obtained from Sigma-Aldrich, Germany, or ThermoFisher Scientific, USA, unless otherwise mentioned.

Superovulation and oocyte collection

CD1 female mice were injected intraperitoneally with 0.1 ml PMSG (50 units/ml, Biovendor, Czech Republic) either alone or combined with mAb-Free α subunit (0.1 ml), followed by an injection of 0.1 ml of hCG (50 units/ml, Sigma-Aldrich, Germany) intraperitoneally 48 hours later. At 17 h after this, mice were sacrificed by CO2 asphyxiation followed by cervical dislocation. The sacrificed animals were dissected, and the oviducts were collected and transferred to the collection dish containing High Calcium HTF medium (Human Tubal Fluid) (Fisher Scientific, USA), pre-equilibrated at 37°C. The clutches of cumulus-oocyte complexes were collected from the oviducts and transferred to a 200-µL drop of fertilization medium containing HTF with 1.25 mM of reduced glutathione (GSH) (Sigma-Aldrich, Germany), covered with paraffin oil (Fisher Scientific, USA), and then incubated at 37°C for 30 min before insemination (Behringer et al., 2014).

***In vitro* fertilization and embryo culture**

Mature male mice were first sacrificed by CO2 asphyxiation and cervical dislocation. The sacrificed animals were dissected, and the cauda epididymis was then collected and placed in a petri dish containing HTF medium. The cauda was punctured using a 26-gauge disposable needle to collect the spermatozoa (Meniru et al., 1998; Nagy et al., 2003). The sperm were first preincubated at 37°C for 10 min to induce capacitation. Then, a total of 20-30µl sperm was slowly collected and expelled gently onto the egg clutches in the fertilization medium drop. The oocytes and sperm were co-cultured in the fertilization HTF-GSH drop for 3 to 4 hours at 37°C with 5% CO2, 5% O2 and 90% N2 incubator. Afterward, the oocytes were washed into three 100µl drops of HTF medium. The number of ovulated oocytes was first counted and then cultured overnight in an HTF medium. At 24h after insemination, the number of 2-cell embryos was counted and the fertilization rates were calculated (Nagy et al., 2003), and the oocytes presenting a fragmented or small ooplasm or an expanded zona pellucida were considered abnormal. The 2-cell embryos were transferred to 100ul drop of Potassium Simplex Optimized Medium (KSOM, Fisher Scientist, USA) for *in vitro* embryo development to the blastocyst stage (Takeo and Nakagata, 2011; Kidder, 2014).

Study design

In this study, the treated group consisted of female CD1 mice receiving PMSG/hCG along with the mAb-Free α subunit, while the control group received only PMSG/hCG.

Effect of immunoneutralization of inhibin-free subunit on ovulation rate

The following investigation was performed in order to examine the immunoneutralization of inhibin-free α subunit on mice ovulation rate. Six replicates were conducted, each consisting of two groups of four female CD1 mice. In each replicate, the treated group received PMSG/hCG with 400 µg of mAb-Free α subunit, while the control group received only PMSG/hCG. A total of 48 female mice were used across all replicates, equally divided into 24 in the treated group and 24 in the control group. Ovulation rates were determined by counting the total number of oocytes produced in each group. Further investigation was also conducted using increased concentrations of mAb-Free α subunit. A total of five groups, each consisting of five female CD1 mice, were used. Four groups received PMSG/hCG in combination with

different doses of mAb-Free α subunit: 200 µg, 400 µg, 800 µg, and 1 mg, respectively. The fifth group, serving as the control, received only PMSG/hCG. Ovulation rates were assessed by counting the total number of oocytes produced in each group.

Effects of immunoneutralization of free inhibin *α* subunit on mice fertilization and embryo development

The following investigation was carried out to examine the effects of immunoneutralization of inhibin-free *α* subunit on *in vitro* fertilization and embryo development. The experiment was repeated three times, involving a total of 22 female CD1 mice and 3 male CD1 mice, with one male used as a sperm donor for each of the following experiments. In each experiment, the mice were divided into treated and control groups. In Experiment 1, 4 mice were treated with PMSG/hCG and 400 µg of mAb-Free *α* subunit, while the 4 control mice received only PMSG/hCG. In Experiment 2, the treated and control groups consisted of 3 mice each. In Experiment 3, 4 mice received PMSG/hCG with the mAb, and 4 mice served as controls, receiving only PMSG/hCG. The oocytes generated from the groups were fertilized with fresh sperm, and *in vitro* embryo culture was then performed. The fertilization rates were calculated as the total number of 2-cell embryos divided by the total number of generated oocytes and multiplied by 100. The embryo development was assessed by the number of 2-cell embryos developed to the blastocyst stage *in vitro*.

Statistical analysis

Results are expressed as the mean ± standard deviation (SD). The significance of the difference between the control groups and the treated groups in all experiments was determined by the Mann-Whitney nonparametric test and relative standard deviation values (RSD). A probability value (P) of less than 0.05 was considered to be significant. RSD values higher than 10% indicated considerable variability, suggesting a significant difference. All statistical analyses were performed using Mini tab software 17.1.0.

To assess the variability across the replicates of the same experiment and evaluate the potential influence of data collection time on ovulation, fertilization, and blastocyst rates, a mixed-effects linear model was employed to account for both fixed and random effects. Treatment and experiment (time) were included as fixed effects, while the random effect was set at the level of the experiment to control for repeated measures over time. The interaction between treatment and experiment was also assessed to determine if the treatment effect varied across different time points. The mixed model was fitted using Restricted Maximum Likelihood (REML) estimation. All statistical analyses were performed using the LME4 package in R (version 4.0.0), with significance evaluated at the 5% level.

RESULTS

The present study investigated the physiological effects of free inhibin *α* subunit on ovulation, *in vitro* fertilization, and embryo development in mice.

Ovulation rate

In the six experiments conducted, a total of 242 oocytes were collected from 24 donors following mAb- free *α* subunit/PMSG/hCG treatment, while a total of 459 oocytes were collected from 24 donors after PMSG/hCG treatment (Table 1, Figure 1). The mean number of oocytes per female obtained from mAb- Free *α* subunit/PMSG/HCG treatment (N=10.08) was lower compared to the PMSG/hCG group (N=19.12), with a statistically significant difference ($p < 0.05$). The results revealed that the neutralization of free inhibin *α* subunit significantly ($p < 0.05$) decreased the ovulation rate in all six experiments by 47.29% ($p < 0.05$) (Figure 2).

The mixed-effects linear model included treatment and experiment (time) as fixed effects, with the experiment also treated as a random effect. The treatment with mAb-free α subunit resulted in a significant decrease in the average number of oocytes per female ($p < 0.05$), while the effects of time (experiment number) and the interaction between treatment and time were not statistically significant ($p > 0.05$).

To further support these findings, a dose-effect study was conducted using increased doses of the mAb against the free inhibin *α* subunit and investigated their effects on the ovulation rate (Table 2). The results showed a significant negative correlation between the mAb concentration injected and the number of oocytes produced indicating that the ovulation rate decreased as a result of increasing mAb concentration (Figure 3, Figure 4), thus supporting the initial findings. According to these findings, it was hypothesized that free inhibin *α* subunit may interfere with the competition system between active and mature inhibin to bind the activin receptor, which involves the α subunit (Figure 5).

Table 1. Effect of treatment with PMSG/hCG + 400ug mAb- Free *α* subunit or PMSG/hCG alone on the ovulation rate of female CD1 aged between 4 and 6 weeks

No. of experiment	Treatment	No. of animals	Total no. of oocyte donors **	Average no. of oocytes/female ± SD
1	PMSG/hCG mAb- Free α subunit	4	33*	8.25 ± 0.94
	PMSG/hCG	4	76	19.00 ± 0.80
2	PMSG/hCG mAb- Free α subunit	4	45*	11.25 ± 0.94
	PMSG/hCG	4	78	19.50 ± 1.28
3	PMSG/hCG mAb- Free α subunit	4	34*	8.50 ± 1.28
	PMSG/hCG	4	80	20.00 ± 0.00
4	PMSG/hCG mAb- Free α subunit	4	42*	10.50 ± 0.56
	PMSG/hCG	4	68	17.00 ± 1.14
5	PMSG/hCG mAb- Free α subunit	4	45*	11.25 ± 0.50
	PMSG/hCG	4	79	19.75 ± 0.64
6	PMSG/hCG mAb- Free α subunit	4	43*	10.75 ± 1.28
	PMSG/hCG	4	78	19.50 ± 2.08

No: Number, SD: Standard deviation. Values are mean ± SD (n = 4). *p < 0.05 versus control group. **The statistical measure of the dispersion of data points around the mean shows low variability between the six experiments for each treatment and high variability between the two groups of treatments.

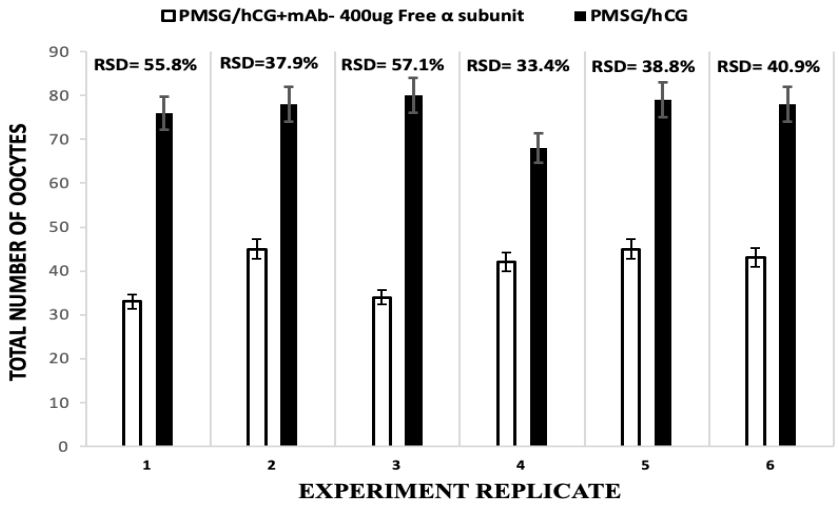

Figure 1. Effects of treatment with PMSG/hCG + 400ug mAb- Free α subunit or PMSG/hCG alone on the ovulation rate of female CD1 with 6 replicates. Number of animals per group was 4 ($p < 0.05$). RSD: Relative standard deviation.

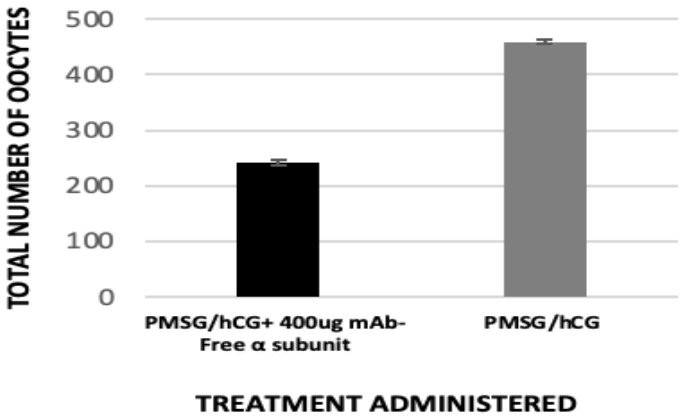

Figure 2. Total number of oocytes generated following two treatments, PMSG/hCG + 400ug mAb- Free α subunit (number of animals was 24) and PMSG/hCG alone (number of animals was 24) in CD1 mice ($p < 0.05$).

Table 2. A dose-effect study using four doses of mAb-Free α subunit administered to CD1 mice on ovulation rate compared with administration of PMSG/hCG alone (n = 5).

Treatment	mAb- Free α subunit concentration	Number of oocyte donors *	Average no. of oocytes/female
PMSG/hCG mAb- Free α subunit	200ug	59	11.80
	400ug	43	8.6
	800ug	30	6
	1mg	26	5.2
PMSG/hCG	--	85	17

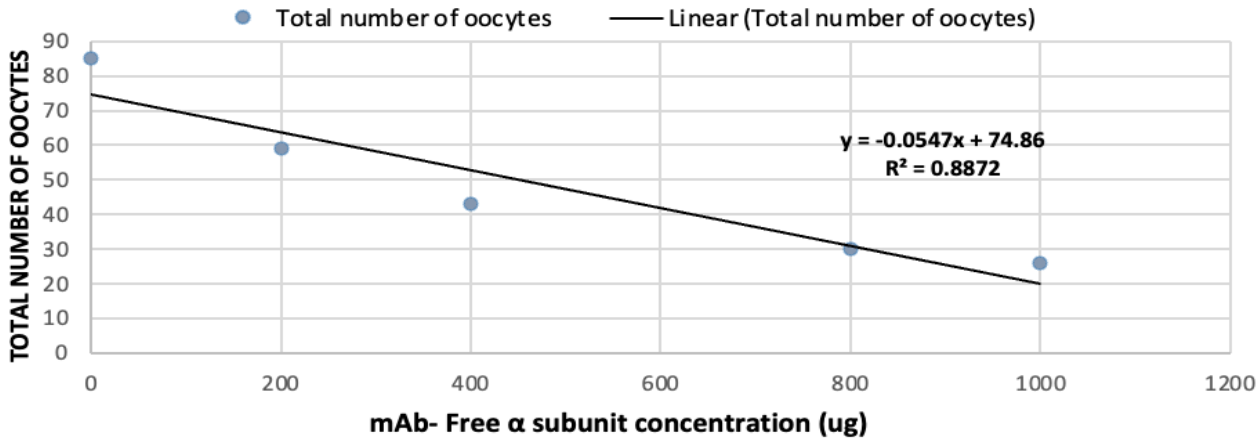

Figure 3. Dose-response regression curve of mAb- Free α subunit concentration on ovulation rate. Number of oocytes collected from CD1 females, One group treated with PMSG/hCG alone and four groups treated with PMSG/hCG combined with increasing concentrations of mAb-Free α subunit, each consisting of 5 animals.

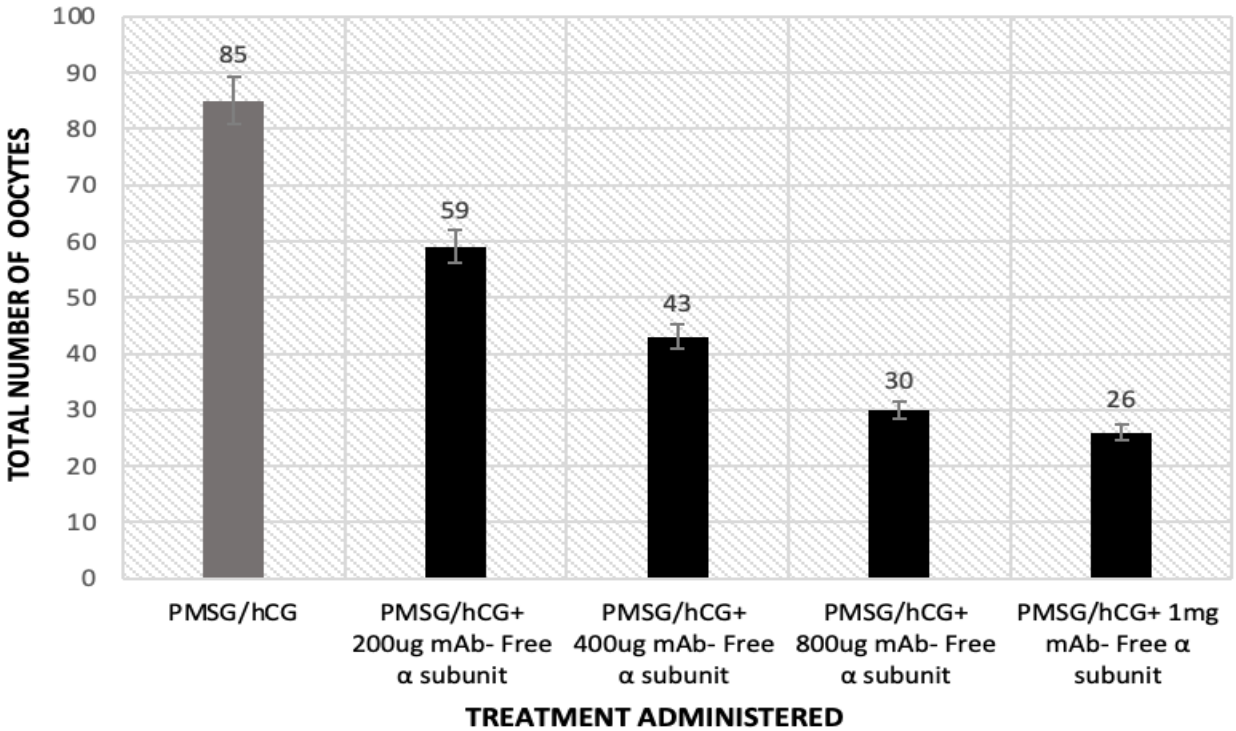

Figure 4. Number of oocytes collected from CD1 females. One group was treated with PMSG/hCG alone, and four groups were treated with PMSG/hCG combined with increasing concentrations of mAb-Free α subunit, each consisting of five animals.

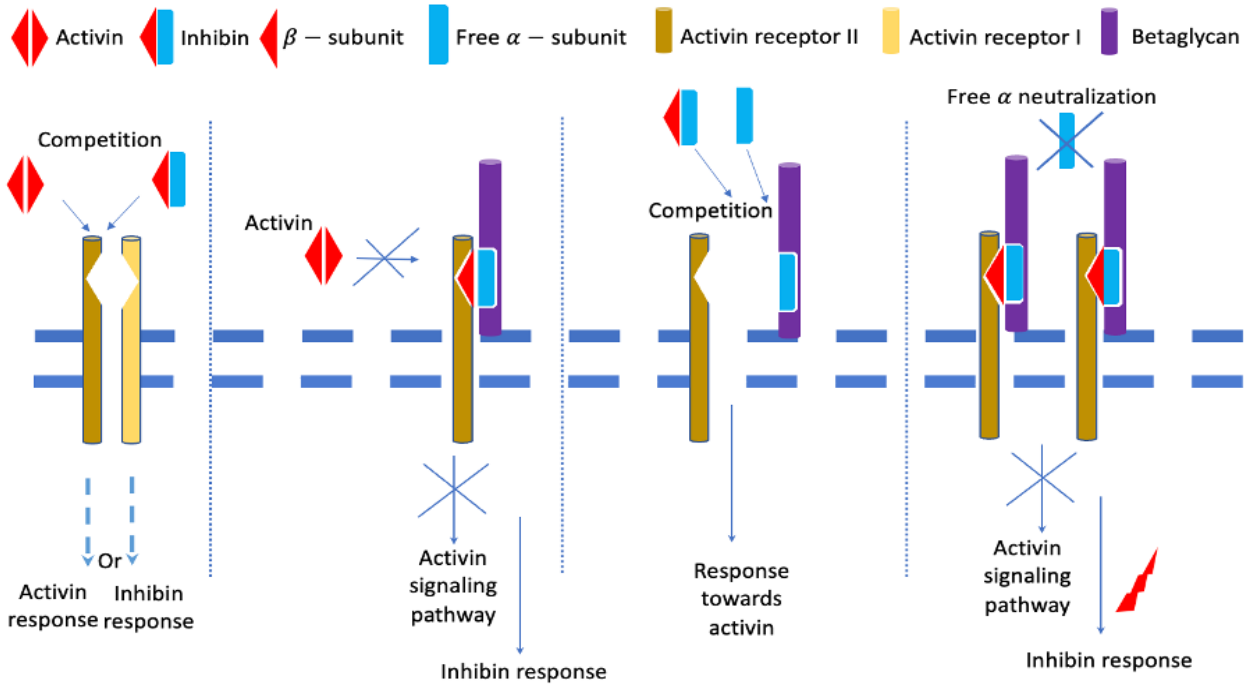

Figure 5. Competition between inhibin and activin β subunits for binding to the activin type II receptor. As a consequence of inhibin binding, activin type I receptor recruitment is inhibited thus blocking activin's action. There is also competition between the α subunit of inhibin and the free inhibin α subunit to bind betaglycan. As a result of the free α subunit binding, inhibin's affinity to the activin type II receptor decreases. Neutralization of the free inhibin α subunit increases the inhibin effect.

Fertilization and embryo development

Further investigation was conducted to examine the effect of the free inhibin α subunit on the fertilization rate of cleaved oocytes as well as the blastocyst development (Table 3). Considering the results of the three experiments, the fertilization rate of mice injected with mAb- free α subunit was 83.48%, as compared to 27.80% in the control group (PMSG/hCG) (Table 3, Figure 6), which represents a significant increase in fertilization of 55.68% ($p < 0.05$). A similar improvement was observed in embryo and blastocyst development following the administration of mAb-free α subunit (Table 3); indeed, in the group treated with mAb- free α subunit, 62.63% of two-cell embryos developed to the blastocyst stage. In the group treated with PMSG/hCG alone, however, only 15.78% of two-cell embryos developed to the blastocyst stage (Figure 7), resulting in a significant increase in the blastocyst rate of 43.85% ($p < 0.05$).

The mixed-effects analysis showed that the treatment with mAb-free subunit had a significant positive effect on both fertilization and blastocyst rates ($p < 0.05$), as compared to the PMSG/hCG treatment alone. However, there was no significant effect of time on either the fertilization rate ($p = 0.593$) or the blastocyst rate ($p = 0.760$), indicating that the timing of the experiments did not significantly influence the outcomes.

Table 3. Effect of treatment with PMSG/hCG + 400ug mAb- Free α subunit (n = 11) or PMSG/hCG alone (n = 11) on the fertilization rate and embryo development of female CD1 aged between 4 and 6 weeks

Number of experiments	Treatment	No of animals	No. of oocyte donors	Average no. of oocytes/female	No. of unfertilized oocytes	No. of two-cell embryos	Fertilization rate (%)	No. of blastocyst	Blastocyst (%)
1	PMSG/hCG mAb- Free α subunit	4	31	7.75	3	28	90.32	17	60.71
	PMSG/hCG	4	80	20	57	23	28.75	3	13.04
2	PMSG/hCG mAb- Free α subunit	3	22	7.33	5	17	77.27	12	70.58
	PMSG/hCG	3	45	15.33	34	11	24.44	2	18.18
3	PMSG/hCG mAb- Free α subunit	4	56	14	10	46	82.14	28	60.86
	PMSG/hCG	4	80	20	57	23	28.75	4	17.39

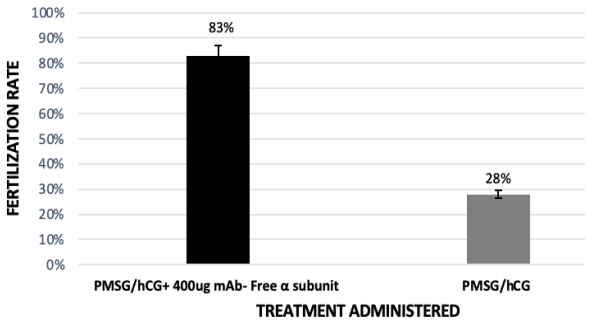

Figure 6. Effects of treatment with PMSG/hCG + 400ug mAb- Free α subunit or PMSG/hCG alone on the fertilization rate of female CD1 (Number of animals per group was 7 e; $p < 0.05$).

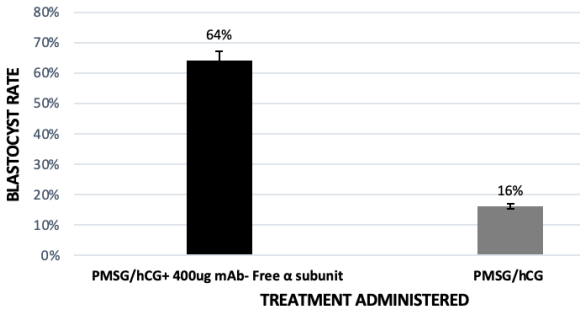

Figure 7. Effects of treatment with PMSG/HCG + 400ug Mab-anti INH α or PMSG/HCG alone on blastocyst and embryo development of female CD1 (Number of animals per group was 7; $p < 0.05$).

DISCUSSION

Inhibin and activin are structurally and functionally related. Unlike inhibin, activin stimulates FSH secretion, and its activity could be indirectly affected by changes in the levels of the free inhibin α subunit (Gray et al., 2005). Inhibin applies its biological effects by antagonizing activin's action (Massagué and Chen, 2000). Activin's binding to the ActRII receptor is critical to initiate a cascade of actions involved in its signaling pathway (Lebrun and Vale, 1997; Kawabata and Miyazono, 1999). Inhibin competes with activin to bind the ActRII receptor via the β subunit, thereby antagonizing activin's signaling (Lebrun and Vale, 1997; Pangas and Woodruff, 2000). The mechanism of action toward activin response or inhibin response depends on the amount of inhibin; at higher concentrations, inhibin is more likely to bind to the ActRII receptor than activin (Martens et al., 1997; Lebrun et al., 1999). Indeed, studies have shown that activin has a higher affinity for the ActRII receptor than inhibin because of avidity/cooperative binding effects, with activin binding two type II receptors at once, while inhibin binds only one (Thompson et al., 2003; Harrison et al., 2004). The study conducted by Lewis et al. (2000) proved the necessity of a co-receptor, betaglycan, to enhance inhibin's affinity to the ActRII receptor, to which inhibin binds via its α subunit (Lewis et al., 2000; Makanji et al., 2008). However, betaglycan has a high affinity for inhibin and can mediate inhibin reactivity to cells that are insensitive to inhibin (Lewis et al., 2000; Esparza-Lopez et al., 2001; Harrison et al., 2001; Brûlé et al., 2021).

The present study assessed the effect of immunoneutralizing the free inhibin α subunit using a novel monoclonal antibody raised against the amino acid epitope HIS^{64} ALA^{65} VAL^{66} GLY^{67} GLY^{68} PHE^{69} MET^{70} HIS^{71} ARG^{72} THR^{73} SER^{74} GLU^{75} PRO^{76} GLU^{77} within the αN region of the mouse inhibin α subunit.

Following the immunoneutralization of the monomeric free inhibin α subunit in mice, the results showed a 47.29% decrease in the ovulation rate compared to the control group ($p < 0.05$; Figure 2, Table 1). Additionally, the ovulation rate decreased further with higher concentrations of the mAb-free α subunit (Figures 3 and 4). These results unexpectedly demonstrated a positive effect of the free inhibin α subunit on follicular development and ovulation in mice, contrasting with the effect of mature inhibin. These findings support the hypothesis addressed by previous studies (Schneyer et al., 1991; Silva et al., 1999; Laird et al., 2019) suggesting that the free inhibin α subunit may have the ability to antagonize inhibin. The effects associated with the production of immunoreactive monomeric pro-αN-αC protein remain an area requiring further research. Evidence suggests that the free inhibin α subunit may possess intrinsic biological properties distinct from those of dimeric inhibins (Risbridger et al., 1989; Lambert-Messerlian et al., 1994; Mason et al., 1996; Drummond et al., 2004; Makanji et al., 2014). It may act as an additional local modulator of follicular function, potentially serving as an inhibin antagonist and thereby functioning as an activin agonist (Silva et al., 1999; Lewis et al., 2000; Chapman and Woodruff, 2003).

To date, experimental research has been insufficient to elucidate the effect of free α subunit on ovulation and embryo development. The monoclonal antibody generated in this study targets the free α subunit of inhibin (~52 kDa) and may influence ovulation and reproductive function through several indirect mechanisms. By neutralizing the free α subunit, it might alter the overall balance and availability of inhibin and activin.

Most superovulation protocols based on inhibin neutralization target inhibin α because it is the common component shared by both inhibin A and B, thereby potentially neutralizing both forms by preventing their proper assembly or receptor interaction. In this study, neutralizing the monomeric free α subunit could potentially alter the processing or availability of mature inhibin forms, which would be expected to enhance, rather than inhibit, ovulation. However, in the presence of its co-receptor betaglycan, inhibin can interact with the activin receptor even at equimolar or lower

concentrations than activin (Carroll et al., 1989; Rivier and Vale, 1991, Weiss et al., 1993; Gray et al., 2005; Brûlé et al., 2021), suggesting that the enhancement of its affinity by betaglycan is more critical for inhibin antagonism.

From another perspective, based on the present results, it can be hypothesized that the free α subunit competes with mature inhibin to bind betaglycan, making ActRII more available to bind activin's β subunit. The neutralization of the free α subunit increases the amount of inhibin binding to betaglycan, thereby increasing activin antagonism (Figure 5). The findings of the present study strongly support this mode of action. Furthermore, a previous study demonstrated that transgenic mice with knockdown of the inhibin α subunit exhibited a 35.28% reduction in litter size, which was associated with a decreased ovulation rate (Kadariya et al., 2015).

Overall, there is widespread agreement that neutralizing endogenous inhibin could enhance folliculogenesis and fertility in females. However, previous research has yielded conflicting results. Numerous studies in both rodents and different farm mammals have clearly demonstrated that active or passive immunization against inhibin or the inhibin α subunit led to an increase in ovulation rate (Wheaton et al., 1996; Mao et al., 2016; Jia et al., 2021; Hasegawa et al., 2022; Mochida et al., 2024). However, other studies did not observe such an increase (Findlay et al., 1989; Ireland et al., 1992; King et al., 1995; Terhaar et al., 1997; Dhar et al., 1998; Lu et al., 2020). According to the findings of the present study, this inconsistency among studies may largely stem from the influence of the free inhibin α subunit, suggesting that in these studies, the antibodies generated after inhibin immunization were likely targeting the free inhibin α rather than the mature α subunit.

In addition to the unique effects of the free α subunit, the mature forms of inhibin A and B have been shown to operate through different mechanisms. They might act through distinct co-receptors to impair activin signaling and suppress FSH secretion and synthesis. A significant amount of data evaluates the effect of immunizing animals against inhibin on serum FSH levels (Drummond et al., 2004). Numerous studies have shown an increase in oocyte numbers following the disruption of inhibin feedback, resulting in elevated FSH levels. However, other studies unexpectedly showed no effect on FSH levels despite stimulated follicular growth and enhanced ovulation. The reasons behind these discrepancies remain unclear. A recent review has analyzed existing models of inhibin action, highlighting how recent discoveries in both mice and humans have posed challenges to these models (Bernard et al., 2020). It has been noted that inhibin A and B elicit distinct reactions within the reproductive system. Despite sharing a common α subunit, they display differing affinities towards betaglycan. Specifically, inhibin A binds to betaglycan with a higher affinity compared to inhibin B. Notably, inhibin B is the form responsible for regulating FSH release during the follicular phase of the menstrual cycle in primates and during metestrus/diestrus in rodents (Bernard and Woodruff, 2001; Chapman and Woodruff, 2003; Yding Andersen, 2017).

Another interesting finding of the present study is the negative effect of the inhibin free α on oocyte maturation and embryo development. This study demonstrates that the neuralization of the free α subunit increases the fertilization rate by 55.68% and the blastocyst development by 43.85%, as compared to the control group ($p < 0.05$, figures 6 and 7), indicating that the free inhibin α subunit may have a negative impact on the quality of the mature oocyte and the competence of the fertilized oocyte to attend the blastocyst stage. The substantial secretion of inhibin by multiple developing follicles may adversely affect follicular development, oocyte function, and quality during follicular atresia and selection (Jimenez-Krassel et al., 2003). Immunoneutralizing inhibin could mitigate the negative impacts of this hormone on maturing oocytes and the resulting embryos. The present study shows a consistent improvement in both embryo yield and development as a result of neutralizing the inhibin free α subunit.

There is substantial evidence to support this finding; a previous study has reported that the mAb against inhibin free α subunit co-cultured with the bovine cumulus oocyte complex enhances the blastocyst yield by 77% (Silva et al., 1999). Furthermore, it has been proved that at higher concentrations, inhibin α subunit is deleterious to embryo development (Fujiwara et al., 2000). In a previous study investigating the influence of progesterone on *in vitro* maturation of bovine oocytes, it was found that the negative impact of progesterone on blastocyst yield was associated with a significantly higher concentration of total inhibin α subunit. They reported that the adverse effect of progesterone on blastocyst yield may be mediated by increased expression of inhibin α subunit by cumulus cells (Silva and Knight, 2000). Furthermore, it has been reported that the presence of free inhibin α subunit during oocyte maturation reduces oocyte development after cleavage (Silva et al., 1999). Results from another study by Li et al. (2011) also demonstrated that the addition of an increased concentration of inhibin antibody decreased the embryo development to blastocyst stage, stating that blastocyst rate was not further enhanced after pretreatment with antibody against inhibin (Li et al., 2011). Together, the present findings revealed that the free inhibin α subunit may have an opposite effect on inhibin at both ovulation and blastocyst rates.

In addition, several studies have investigated the impact of inhibin immunoneutralization as a superovulation protocol on oocyte quality and subsequent blastocyst formation after *in vitro* fertilization, studies revealing varying outcomes. Some have demonstrated that superovulation treatment can negatively affect both fertilization rates and

embryo development (Takeo and Nakagata, 2015; Hasegawa et al., 2016), while others have found no significant differences compared to the control groups (Wang et al., 2001; Ishigame et al., 2004). Further insights were gained in studies that administered increased doses of anti-inhibin treatment, which revealed a greater decrease in blastocyst development, suggesting a negative correlation (Wang et al., 2001; et al., 2004; Li et al., 2011).

CONCLUSION

In summary, the present study demonstrated that the free inhibin α subunit has a positive effect on ovulation rate in contrast to the effect of mature inhibin. However, the free inhibin α subunit was shown to harm embryo competence. These findings provided experimental evidence to support the hypotheses and questions raised by various studies regarding the potential antagonist effect of inhibin-free α on inhibin itself. Together, these results indicated that free inhibin α subunit and inhibin protein may play an opposite effect in normal ovarian physiology. In so far as the present study was conducted only on mice, further investigation should be conducted to validate these observations in other mammalian species. Furthermore, the findings of the present study may open a new area of investigation in understanding the physiological effects of inhibin and its unique signaling pathways.

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Availability of data and materials

The datasets generated during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

Kaoutar Aalilouch, Ouafaa Fassi Fihiri, Khalida Sabeur, Faouzi Kichou, and Ikhlass El Berbri designed and coordinated the work. Najet Safini and Mehdi Elharrak conducted the epitope design and supervised the antigen immunization for antibody production. Kaoutar Aalilouch performed the cell fusion and monoclonal antibody production and purification under the supervision of Ouafaa Fassi Fihri. Kaoutar Aalilouch and Khalida Sabeur evaluated the *in vitro* and *in vivo* activity of the produced antibody, conducting laboratory work on mice ovulation tests, IVF, and embryo development. The manuscript was written by Kaoutar Aalilouch. Kaoutar Aalilouch conducted the presented statistical analysis of the experimental data, with contributions from Khalida Sabeur and Ikhlass El Berbri. All authors participated in the overall interpretation of the data and confirmed the final draft of the manuscript for submission to the journal.

Competing interests

The authors declare that they have no competing interests

Ethical considerations

The authors confirm that all authors have reviewed and submitted the original manuscript to this journal for the first time.

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