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Role of FSH glycan structure in the regulation of Sertoli cell inhibin production

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Short Title: FSH oligosaccharides and inhibin production

Abstract

Variations in FSH carbohydrate composition and structure are associated with important structural and functional changes in Sertoli cells (SCs) during sexual maturation. The aim of the present study was to investigate the impact of FSH oligosaccharide structure and its interaction with gonadal factors on the regulation of monomeric and dimeric inhibin production at different maturation stages of the Sertoli cell. Recombinant human FSH (rhFSH) glycosylation variants were isolated according to their sialylation degree (AC and BA) and complexity of oligosaccharides (CO and HY). Native rhFSH stimulated inhibin α-subunit (Pro-αC) but did not show any effect on inhibin B (InhB) production in immature SCs isolated from 8-day-old rats. Activin A stimulated InhB and had a synergistic effect on FSH to stimulate Pro-αC. The less acidic/sialylated rhFSH charge analogues, BA, were the only charge analogue mix that stimulated InhB as well as the most potent *stimulus* for Pro-αC production. Native rhFSH stimulated both Pro-αC and InhB in SCs at a more advanced maturation stage, isolated from 20-day-old rats. In these cells, all rhFSH glycosylation variants increased InhB and Pro-αC production, even in the presence of growth factors. The BA preparation exerted a more marked stimulatory effect on InhB and Pro-αC than the AC. Glycoforms bearing high mannose and hybrid-type oligosaccharides, HY, stimulated InhB and Pro-αC more effectively than those bearing complex oligosaccharides, CO, even in the presence of gonadal growth factors. These findings demonstrate the modulatory effect of FSH oligosaccharide structure on the regulation of inhibin production in the male gonad.

Introduction

The Sertoli cell plays a central role in the development of a functional testis, providing the structural and nutritional support for germ cells. The Follicle-stimulating hormone (FSH) controls Sertoli cell function; supports cell proliferation prior to puberty, participates in cell maturation and regulates the synthesis of Sertoli cell-derived products which are essential for germ cell survival and function in the adult testis (Meachem *et al*., 2005).

Like other glycoprotein hormones, FSH consists of a family of glycosylation variants which differ from each other in their oligosaccharide composition including degree of branching and sialylation (Ulloa-Aguirre *et al*., 1995a).

The hormonal milieu regulates the synthesis and secretion of FSH glycosylation variants (Ulloa-Aguirre *et al*., 1999; 2001). The Gonadotrophin-releasing hormone (GnRH) and sexual steroids are recognized endocrine factors involved in the regulation of FSH microheterogeneity in females and males (Padmanabhan *et al*., 1988a; 1988b; Ulloa-Aguirre *et al*., 1986; 2001; Creus *et al*., 1996; Wide, 1989; Simoni *et al*., 1992). Previous studies carried out in normal women showed that the endocrine milieu modulates both the incorporation of the terminal sialic acid residue and the complexity of the carbohydrate chain. Differences in these two characteristics of FSH molecular microheterogeneity have been observed during lactational amenorrhoea and in postmenopausal women when compared with the midfollicular phase of ovulatory cycles (Creus *et al*., 1996; Velasquez *et al*., 2006).

In normal boys, changes in FSH microheterogeneity have been described during pubertal development, which is characterized by the presence of a growing proportion

of FSH glycoforms with complex oligosaccharides and transient variations in the sialylation degree in circulation (Phillips *et al*. 1997; Olivares *et al*., 2004; Campo *et al*., 2007). Similar changes have been observed during sexual development in the male rat (Chappel & Ramaley, 1985; Rulli *et al*., 1999; Ambao *et al*., 2009). However, the possible impact that FSH oligosaccharide structure may have on the regulation of Sertoli cell endocrine activity has never been explored.

During the last years, inhibin B and anti-Müllerian hormone (AMH) have been considered reliable markers of Sertoli cell function (Lahlou & Roger, 2004; Trigo *et al*., 2004; Bergadá *et al*., 2008; Grinspon *et al*., 2012; 2013; Rey *et al*., 2013). Although production of these two peptides is regulated by FSH, inhibin B serum profile showed that adult levels of this peptide are present in circulation during the first years of life concomitantly with prepubertal FSH concentrations (Andersson *et al*., 1998; Byrd *et al*., 1998). Therefore, immature Sertoli cells have a great capacity to produce inhibin B independently of FSH *stimulus* and in the absence of spermatogenesis (Bergadá *et al*., 1999; 2006).

The aim of the present study was to investigate the impact of FSH oligosaccharide structure and its interaction with gonadal factors on the regulation of monomeric and dimeric inhibin production at different stages of Sertoli cell maturation.

Materials and methods

Reagents

rhFSH was purchased from the National Hormone and Peptide Program of the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK)-NIH (Torrance, CA, USA). Insulin, TGF-β1, EGF, rat IL1-β and transferrin were purchased from Sigma-

Aldrich, Inc. (St. Louis, MO, USA). Tissue culture media were obtained from Gibco® by Thermo Fisher Corporation (Carlsbad, CA, USA). All other chemicals were of reagent grade from standard commercial sources.

Animals

Sprague-Dawley rats were obtained from the animal care unit of the Instituto de Biología y Medicina Experimental (IBYME-CONICET, Buenos Aires, Argentina). Eight or 20-day-old male rats were euthanized by asphyxiation with CO_2 and decapitation or cervical dislocation, respectively, and the testes were removed for Sertoli cell isolation. All experimental procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the local Institutional Ethic Committee (IBYME-CONICET).

Rat Sertoli cell isolation and culture

Sertoli cells from 8-day-old Sprague-Dawley rats were isolated as previously described (Scheingart *et al*., 1995; Galardo *et al*., 2008). Briefly, decapsulated testes were incubated in culture medium containing 0.03% collagenase and 0.003% soybean trypsin inhibitor for 5 min at room temperature. Culture medium consisted of a 1:1 mixture of Ham's F12 and DMEM, supplemented with 0.1% bovine serum albumin, 100 IU/mL penicillin, 2.5 µg/mL amphotericin B and 1.2 mg/mL sodium bicarbonate. After the initial dispersion, seminiferous tubules were sedimented and supernatant was discarded to remove interstitial cells. When indicated, seminiferous tubules were submitted to an extra 1 M glycine-2 mM EDTA (pH 7.4) treatment to remove peritubular cells. After several washes, a second collagenase treatment was performed. Tubules were treated for 10 min at room temperature with a solution of 0.03% collagenase,

105 0.003% soybean trypsin inhibitor and 0.03% DNAse. The Sertoli cell suspension,
106 collected by sedimentation, was resuspended in the culture medium described above
107 with the following additions: 10 µg/mL transferrin, 5 µg/mL insulin, 5 µg/mL vitamin E
108 and 4 ng/mL hydrocortisone. Sertoli cells were cultured in 24-multiwell plates (15 µg
109 DNA/cm^2) at 34 °C in a mixture of 5% CO_2:95% air. The proportion of peritubular myoid
110 cells present in the immature Sertoli cell cultures was in the range of 10-15%, as
111 evaluated by α-mooth muscle actin (α-SMA) immunostaining. When the extra glycine-
112 EDTA treatment was applied during the cell isolation procedure, this proportion
113 decreased to 3-5%.

114 Sertoli cells from 20-day-old Sprague-Dawley rats were isolated as previously
115 described (Meroni *et al*., 1999; 2002). Briefly, decapsulated testes were digested with
116 0.1% collagenase and 0.006% soybean trypsin inhibitor in Hanks' balanced salt solution
117 for 5 min at room temperature. Seminiferous tubules were saved, cut and submitted to 1
118 M glycine-2 mM EDTA (pH 7.4) treatment to remove peritubular cells. The washed
119 tubular pellet was then digested again with collagenase for 10 min at room temperature
120 to remove germinal cells. The Sertoli cell suspension, collected by sedimentation, was
121 resuspended in culture medium which consisted of a 1:1 mixture of Ham's F-12 and
122 DMEM, supp lemented with 20 mM HEPES, 100 IU/mL penicillin, 2.5 µg/mL
123 amphotericin B, 1.2 mg/mL sodium bicarbonate, 10 µg/mL transferrin, 5 µg/mL insulin, 5
124 µg/mL vitamin E and 4 ng/mL hydrocortisone. Sertoli cells were cultured in 24-multiwell
125 plates (5 µg DNA/cm^2) at 34 °C in a mixture of 5% CO_2:95% air. No peritubular myoid
126 cell contamination was revealed in the cultures when an immunoperoxidase technique
127 was applied to Sertoli cell cultures using a specific anti-α-SMA antibody. Germ cell

contamination was below 5% after 48 h in culture as examined by phase-contrast microscopy.

Sertoli cells were allowed to attach for 48 h in the presence of insulin and medium was replaced at this time with fresh medium without insulin. As from day 3, the cells were incubated 24 (8-day-old) or 72 h (20-day-old) in the absence or presence of the different stimuli, as indicated in the Figure legends.

For cAMP determination, immature Sertoli cells were incubated in the presence of 0.1mM IBMX and media were collected after 90 min of treatment. To evaluate aromatase activity, 2 mM 19-hydroxyandrostenedione as substrate for aromatization were added on the fourth day.

Following incubation, Sertoli cells were stored at -80 °C until DNA determination and conditioned media were stored at -20 °C until hormone measurements were carried out. Total DNA was determined by the method of Labarca & Paigen (1980).

Peritubular cell isolation and culture

In order to isolate peritubular cells, 8 day-old-rat seminiferous tubule fragments, obtained after the initial dispersion, were seeded at low density (5 μg DNA/cm^2) in the presence of 10% FCS. After 7 days, the peritubular cell monolayer was collected by trypsin/EDTA treatment and seeded in 12-multiwell plates. Cells were allowed to attach and grow for 72 h and medium was replaced at this time with fresh medium without serum.

Isolation of rhFSH glycosylation variants

Glycosylation variants were isolated from rhFSH according to either their sialylation degree or oligosaccharide complexity using preparative isoelectric focusing

(IEF) and lectin (concanavalin A, ConA) affinity chromatography, respectively. Two native rhFSH ampoules (40 mg LER-907) were applied for each procedure. The content of each ampoule was dissolved in double-distilled and deionized water (Barnstead NANOPure II, Thermo Scientific, Baltimore, MD, USA) and applied into a preparative IEF cell or into a ConA column.

Preparative IEF

Preparative IEF was used to isolate rhFSH charge analogues according to their sialylation degree using a Rotofor system (Rotofor Preparative Cell, Bio-Rad Laboratories, Inc.) as described previously (Bedecarras *et al*., 1998; Loreti *et al*., 2009; 2013). Focusing was carried out at 12W constant power (Power Pac 3000 Bio-Rad Laboratories, Inc.) for 4h, maintaining the chamber refrigerated (Refrigerated Circulator, Forma Scientific, Inc., Marietta, OH, USA). Twenty fractions (2.5 mL each), from a 3-10 pH gradient, were harvested and their pH was determined. Each individual fraction was exhaustively dialysed against 1 M NaCl to completely eliminate ampholytes and detergent and I-FSH content was determined by double-antibody RIA. The rhFSH recovery range was 70–85%.

Based on the variations observed in the proportion of FSH isolated at the opposite ends of the pH gradient, associated with changes in testicular activity (Campo *et al*., 2007; Ambao *et al*., 2009), two rhFSH preparations were selected to explore the biological effect of their sialylation degree. Fractions from pH 2.56 to 4.00 (more acidic/sialylated charge analogues mix; rhFSH-AC) and at pH >5.00 (less acidic/sialylated preparation; rhFSH-BA) were combined, concentrated using

Centriprep-10 membrane (cut-off 10 000; Amicon, Beverly, MA, USA) and stored at -80 °C.

ConA chromatography

ConA chromatography was used to isolate rhFSH glycosylation variants according to the complexity of their oligosaccharides, as previously described by Creus *et al*. (1996). The isolation of three groups of glycoforms was based on the different affinities that the carbohydrate structures have for this particular lectin. Briefly, equilibrium buffer (50 mM Tris–HCl; pH 7.4, 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM $MnCl_2$) was used to elute unbound rhFSH glycoforms: bearing complex, triantennary and bisecting oligosaccharides (rhFSH-CO); equilibrium buffer containing 10 mM methyl-α-D-glucopyranoside (glucoside) was used to elute the weakly bound rhFSH glycoforms: bearing biantennary carbohydrate chains; and equilibrium buffer containing 0.1 M methyl- α - D-mannopyranoside (mannoside) was used to elute the firmly bound rhFSH glycoforms: bearing high mannose/hybrid-type oligosaccharides (rhFSH-HY). The procedure was carried out at 4 °C. Eluates containing rhFSH glycoforms were dialysed against 0.01 M NaCl, concentrated (Centriprep membranes, Amicon) and stored at -80 °C. The range of recovered rhFSH was 75–90%.

Two rhFSH preparations (rhFSH-CO and rhFSH-HY) were used to explore the biological effect of gonadotrophin oligosaccharide complexity on Sertoli cell function. They were selected based on the previously observed variations in the relative proportion of these two types of types of glycosylation variants when determined throughout sexual development in boys and male rats (Campo *et al*., 2007; Rulli *et al*., 1999).

196 ***Hormone measurements***

197 The rhFSH content of samples was measured using an in-house double-antibody
198 RIA with reagents provided by NIDDK (Bethesda, MD, USA). The RP LER-907 (1 mg
199 LER-907Z 53 IU Second International Reference Preparation, hMG) was used to
200 construct the standard curve. The polyclonal antibody, anti-hFSH-6, was used as
201 antiserum. Purified hFSH (hFSH-ISIAFP-1) was iodinated using the chloramine-T
202 method (Greenwood *et al*., 1963). To minimize the effects of interassay variations, as
203 well as to determine the degree of parallelism between the unknown samples and the
204 FSH standards, all glycoform preparations were analyzed at multiple dose levels in the
205 same assay run. The intra- and interassay coefficients of variation were < 9 and 12%
206 respectively. Simultaneous curve fitting of the dose–response curves obtained in the
207 RIA of the glycoforms revealed no significant differences among the slopes generated
208 by the standard LER-907 and the different rhFSH glycosylation variants, suggesting that
209 the glycoforms were equally recognized by the antibody. rhFSH is expressed in terms of
210 hFSH-2 standard (NIDDKNHPP).

211 Oestradiol was determined by RIA as previously described (Escobar *et al*.,
212 1976). Intra-and interassay coefficients of variation were 8% and 15%, respectively.

213 Dimeric inhibins A and B, free inhibin α-subunit (Pro-αC) and total activin A levels
214 in the culture media were measured using specific two-site enzyme-linked
215 immunoabsorbent assays (ELISA) (Oxford Bio-Innovation Ltd, Oxon, UK) as described
216 previously (Groome *et al*., 1994; 1995; 1996; Knight *et al*., 1996). Recombinant human
217 inhibin A and B and activin A (Genentech, San Francisco, CA, USA), and a partially
218 purified (>75% purity) Pro-αC preparation were used as standards. The assay

sensitivity was 7 pg/mL for inhibin A, 15 pg/mL for inhibin B, 2 pg/mL for Pro-αC and 78 pg/mL for activin A. Activin A, activin B, and follistatin had less than 0.1% cross-reaction in dimeric inhibin assays. Inhibin A had less than 0.5% cross-reaction in the inhibin B assay whereas inhibin B had less than 0.1% cross-reaction in the inhibin A assay. The Pro-αC assay had less than 0.1% cross reactivity with inhibin A, B, activin A, B and follistatin. The total activin A assay had less than 0.5% cross-reaction with inhibin A, inhibin B or activin B, less than 0.1% cross-reaction with bovine Pro-αC or follistatin and only a small (1-5%) cross reaction with activin AB. Intra- and interassay coefficients of variation were less than 10% for all four assays. The human inhibin A and B assays had been validated and successfully used in the rat (Lanuza *et al*., 1999; Arai *et al*. 2002).

Determination of cyclic AMP production

Immature Sertoli cells were cultured for 90 min in the presence of 0.1mM IBMX (specific inhibitor of phosphodiesterase activity) with or without the indicated doses of native rhFSH. The extracellular content of cAMP was determined by a commercial RIA (Immunotech, Beckman Coulter, CA, USA). The sensitivity of the assay was 0.06 nM and the intra- and interassay coefficients of variation were less than 10%.

Statistical analysis

All experiments were run in triplicates and repeated at least three times. Data are expressed as mean ± SEM. Data were log transformed before statistical analysis when appropriate. Comparisons between treatments were carried out using parametric or non-parametric unpaired *t*-test or one-way analysis of variance (ANOVA) followed by multiple comparisons test as appropriate. Differences were considered significant at

241 *P*<0.05. All statistical analyses were performed using GraphPad Prism version 5.00 for
242 Windows (GraphPad Software, San Diego, CA, USA. www.graphpad.com).

243 **Results**

244 Sertoli cells isolated from 8-day-old rats produced 53.3 ± 3.9 pg/μg DNA of
245 inhibin B, 21.4 ± 0.7 pg/μg DNA of activin A and 3.40 ± 0.67 pg/μg DNA of inhibin α-
246 subunit (Pro-αC) when cultured under basal conditions for 24h.

247 Under identical experimental conditions inhibin B and activin A were not detected
248 in the conditioned media of Sertoli cells isolated from 20-day-old rats. When the culture
249 period was prolonged to 72h, they produced 33.4 ± 4.9 pg/μg DNA of inhibin B, 13.1 ±
250 1.4 pg/μg DNA of activin A and 13.8 ± 1.5 pg/μg DNA of Pro-αC.

251 Based on these results, further studies on 20-day-old rat Sertoli cells were
252 performed after 72h of culture.

253 Inhibin A was always undetectable in Sertoli cell cultures.

254 ***Effect of native rhFSH, its glycosylation variants and gonadal factors on inhibin***
255 ***production by Sertoli cells isolated from 8-day-old rats***

256 Production of Pro-αC was stimulated by rhFSH in a dose-dependent manner.
257 The lowest dose used (0.08 ng/mL) was able to significantly stimulate the production of
258 this peptide: 2.71 ± 0.29-fold increase over basal (P<0.05). The highest doses used (2.5
259 and 5 ng/mL) exerted a similar response: 7.88 ± 0.68 vs 8.24 ± 0.54-fold increase over
260 basal (NS) (Fig. 1, panel A).

261 Native rhFSH did not induce changes in basal inhibin B production at any of the
262 doses used in this study (Fig. 1, panel A).

The production of cAMP and oestradiol was evaluated in order to confirm Sertoli cell ability to respond to native rhFSH stimulus under these experimental conditions (Fig. 1, panel B). A dose-dependent response on cAMP production was observed after native rhFSH stimulation (2.3- to 23.0-fold increase over basal, $P<0.01$). Oestradiol production was also stimulated by native rhFSH in a dose-dependent manner; a significant increase was observed at the dose of 0.16 ng/mL (1.53 ± 0.17-fold increase over basal, $P<0.05$). When 2.5 and 5 ng/mL doses of rhFSH were added to the culture, there was no significant variation in the response: 5.09 ± 0.41 vs 5.25 ± 0.37-fold increase over basal, NS) (Fig. 1, panel B).

Based on these results, the selected doses of rhFSH glycosylation variants chosen to assess possible differential biological effects on Sertoli cells were 0.16 and 2.5 ng/mL.

To evaluate the influence of the rhFSH sialylation degree on monomeric and dimeric inhibin production, two preparations of rhFSH charge analogues were used to stimulate immature Sertoli cells. Figure 1 (panels C and D) shows the effect of more acidic/sialylated (AC) and less acidic/sialylated (BA) rhFSH charge analogues added to 8-day-old rat Sertoli cell cultures.

No changes were observed in inhibin B production when the lowest dose of the two charge analogue preparations was used; whereas at the dose of 2.5 ng/mL inhibin B production was significantly stimulated by the less sialylated charge analogue preparation: 1.92 ± 0.09-fold increase over basal ($P<0.01$) (Fig. 1, panel C).

Both rhFSH charge analogue preparations significantly stimulated Pro-αC production, even at the lowest dose used (Fig. 1, panel D). A differential effect was

induced by the less sialylated charge analogue preparation when compared to the more sialylated counterparts. There was a sharper increase of this peptide production when BA rhFSH was added to the culture; 0.16 ng/mL: AC, 2.23 ± 0.27 vs BA 7.62 ± 0.80 and 2.5 ng/mL: AC, 7.54 ± 0.72 vs BA 17.02 ± 1.21 -fold increase over basal ($p<0.05$).

The influence of the rhFSH oligosaccharide complexity on inhibin production was also evaluated. When rhFSH glycoforms bearing high mannose and hybrid-type oligosaccharides (HY) or bearing complex oligosaccharides (CO) were added to the cultures no consistent effect was observed in inhibin B production. However, both preparations stimulated Pro-αC at the 2.5 ng/mL dose ($p<0.05$); a more marked effect was observed when the rhFSH glycosylation variants bearing incomplete oligosaccharides were used (CO, 7.62 ± 0.74 vs HY, 22.59 ± 3.97- fold increase over basal, $p<0.05$).

The ability of 8-day-old-rat Sertoli cells to produce considerable amounts of inhibin B under basal conditions after 24h of culture and the lack of response to native rhFSH led us to further investigate other possible mechanisms involved in the regulation of this dimer production. For this purpose the effect of activin A was evaluated; it induced a significant stimulation on inhibin B and Pro-αC production: 1.32 ± 0.03 and 3.67 ± 0.58-fold increase over basal, respectively ($P<0.05$)*;* when follistatin was added to the culture, the production of both forms of inhibin was significantly reduced: 53% and 69%, respectively ($p<0.05$) (Fig. 2, panels A and B).

An additional step in the isolation process in order to significantly reduce the presence of peritubular cells in the culture was performed. Under these experimental conditions a significant decrease in inhibin B production was observed (42%, $p<0.05$)

(Fig. 2, panel C). Concomitantly, the Sertoli cell culture depleted of peritubular cells ($S_{8\text{-}Gly}$) showed a 61% decrease in the production of activin A ($p<0.001$, Fig. 2 panel E). The response of $S_{8\text{-}Gly}$ cells to the addition of exogenous activin A was as expected; inhibin B production was markedly stimulated: 1.61 ± 0.07-fold increase over $S_{8\text{-}Gly}$, ($P<0.01$) (Fig. 2, Panel C).

The addition of activin A induced a marked effect in inhibin α-subunit production; a 4.68 ± 0.59-fold increase over basal was observed in $S_{8\text{-}Gly}$ cell culture, ($P<0.01$; Fig. 2, panel D).

Interestingly, isolated peritubular cells in culture produced activin A (102 ± 13 pg/µg DNA) but were unable to produce inhibin B (Fig. 2, panel F).

The effect of combined activin A and rhFSH on inhibin B production was evaluated in order to recreate the in vivo situation. A similar increment was observed when the two hormones were added; both individually and combined (1.32 ± 0.03 vs 1.51 ± 0.02-fold increase over basal, $P>0.05$) (Fig. 3, panel A).

The response in terms of Pro-αC production was slightly different since a synergic effect was induced by the combination of both hormones; in that condition a marked increment was observed 32.2 ± 0.6 vs 3.67 ± 0.58 -fold increase over basal ($P<0.001$) (Fig. 3, panel B).

Effect of native rhFSH, its glycosylation variants and gonadal factors on inhibin production by Sertoli cells isolated from 20-day-old rats

Increasing doses of rhFSH (0.1 - 50 ng/mL) were used to assess its effect on Sertoli cell inhibin production. Pro-αC was stimulated by rhFSH in a dose-dependent manner; at the lowest dose (0.1 ng/mL) it increased 1.38 ± 0.06-fold over basal

(P<0.05) and at the highest dose (50 ng/mL) 12.72 ± 0.56-fold over basal (p<0.001) (Fig. 4, panel A).

Native rhFSH was able to stimulate inhibin B production at the dose of 1 ng/mL (1.95 ± 0.15-fold increase over basal, p<0.05); the 10 ng/mL dose increased inhibin B production 2.76 ± 0.21-fold over basal (p<0.001); higher doses of the gondotrophin did not induce further increments (Fig. 4, panel A).

The effect of more acidic/sialylated (AC) or less acidic/sialylated (BA) rhFSH charge analogues and glycoforms bearing high mannose and hybrid-type oligosaccharides (HY) or bearing complex oligosaccharides (CO), at two different doses (1 and 10 ng/mL), on 20-day-old rat Sertoli cells inhibin production was evaluated.

Both preparations of rhFSH charge analogues stimulated monomeric and dimeric inhibin (p<0.05, Fig. 4, panels B and C). However, less acidic/sialylated charge analogues stimulated inhibin B and Pro-αC production more markedly than the more acidic ones at both doses used (1 and 10 ng/mL): inhibin B, 1.79 ± 0.13 vs 1.24 ± 0.07 and 2.43 ± 0.09 vs 1.76 ± 0.16-fold increase over basal; Pro-αC: 4.52 ± 0.21 vs 2.22 ± 0.20 and 9.10 ± 0.36 vs 5.53 ± 0.44-fold increase over basal (BA vs AC, respectively, p<0.05).

Both rhFSH glycosylation variants isolated according to oligosaccharide complexity significantly stimulated inhibin B and Pro-αC production at 1 and 10 ng/mL (p<0.05, Fig. 4, panels D and E). Glycoforms bearing high mannose and hybrid-type oligosaccharides stimulated inhibin B and Pro-αC production more effectively than those bearing complex oligosaccharides: inhibin B: 2.11 ± 0.17 vs 1.31 ± 0.08 and 2.78 ± 0.11

vs 1.86 ± 0.15 -fold increase over basal; Pro-αC: 6.21 ± 1.34 vs 2.66 ± 0.61 and 10.75 ± 1.85 vs 5.79 ± 0.64-fold increase over basal (HY vs CO, respectively, $p<0.05$).

The effect of gonadal factors on inhibin and activin A production was evaluated both individually and in combination with native rhFSH and its glycosylation variants.

When the effect of combined TGF-β, EGF and insulin was evaluated an enhancement in inhibin B, Pro-αC and activin A production was observed (1.41 ± 0.13; 2.18 ± 0.36 and 1.56 ± 0.25-fold increase over basal, respectively, $p<0.05$). In the presence of these growth factors, a further stimulation was elicited by rhFSH on inhibin B and Pro-αC production (2.80 ± 0.18 vs 1.41 ± 0.13 and 14.81 ± 0.90 vs 2.18 ± 0.36 -fold increase over basal, respectively, $p<0.05$, Fig. 5, panels A and B). In contrast, the addition of rhFSH combined with the gonadal factors not only abolished the increment of activin A production induced by TGF-β, EGF and insulin, but also reduced its levels by 46% below the basal condition ones ($p<0.05$); when the effect of native rhFSH was evaluated a 32% reduction of the basal production was observed ($p<0.05$).

Based on the differential effect on inhibin production induced by rhFSH oligosaccharide structure, both its sialylation degree and its complexity, we further evaluated the influence of rhFSH glycosylation variants in the presence of gonadal factors.

When the effect of rhFSH sialylation degree on inhibin B and Pro-αC production was evaluated in the presence of combined TGF-β, EGF and insulin, both rhFSH charge analogues preparations exerted a similar stimulation (2.85 ± 0.22 vs 2.93 ± 0.28 and and 17.06 ± 0.54 vs 19.44 ± 1.70-fold increase over basal, AC vs BA, respectively, $p>0.05$) (Fig. 5, panels C and D).

As shown in Fig. 5, the differential effect induced by the complexity of rhFSH oligosaccharides on inhibin B and Pro-αC production was maintained even in the presence of gonadal factors (2.66 ± 0.22 vs 3.48 ± 0.33 and and 11.68 ± 1.01 vs 20.04 ± 3.64-fold increase over basal, CO vs HY, respectively, $p<0.01$) (Fig. 5, panels E and F).

Discussion

The differential actions of FSH glycosylation variants on granulosa cell function and follicular development have been previously reported (Ulloa-Aguirre *et al*., 1995b; Vitt *et al*., 1998; Nayudu *et al*., 2002). Moreover, it has been demonstrated that FSH microheterogeneity exerts a differential regulation on granulosa cell inhibin production and its global gene expression (Loreti *et al*., 2013a; 2013b).

Important structural and functional changes occur in Sertoli cells during the maturation process. The present study was aimed at elucidating new regulatory mechanisms of monomeric and dimeric inhibin production at different stages of Sertoli cell maturation.

The results obtained herein describe for the first time the differential effect of rhFSH and its glycosylation variants as well as the interaction with gonadal factors produced by other testicular cell types on monomeric and dimeric inhibin production at different stages of Sertoli cell maturation. Cultured immature Sertoli cells isolated from 8-day-old rats and cells in the process of terminal maturation obtained from 20-day-old rats were used.

The production of oestradiol stimulated by FSH is characteristic of the immature Sertoli cell endocrine activity. This ability gradually decreases with age and disappears

when the cells complete their maturation process (Dorrington *et al*., 1978; Rommerts *et al*., 1982; Tsai-Morris *et al*., 1985; Le Magueresse & Jegou, 1988). Previous studies performed on cultured immature Sertoli cells showed that the FSH sialylation degree was inversely related to its biopotency in terms of oestradiol production; pituitary human FSH glycosylation variants isolated at pH>5 elicited the most marked stimulatory effect (Creus *et al*., 2001).

In the present study, outstanding features of immature Sertoli cells in culture were the high capacity of these cells to produce inhibin B under basal conditions and the lack of response to native rhFSH stimulus. However, these cells were highly sensitive to the action of the gonadotrophin to produce oestradiol and inhibin α-subunit in a dose-dependent manner.

Based on the above mentioned results regarding the high oestradiol production induced by hFSH charge analogues isolated at pH>5 on immature Sertoli cells, we assessed the possible relevance of differences in rhFSH sialylation degree on inhibin production. The less acidic/sialylated rhFSH charge analogues (BA) resulted in a more potent stimulus than the more acidic/sialylated ones (AC) for inhibin α-subunit production. An aspect worthy of mention was the effect of the less acidic/sialylated rhFSH charge analogues on the inhibin B production, since native rhFSH did not change basal levels.

These results indicate that the important inhibin B production, characteristic in these cells, can only be further increased by a hormone practically devoid of sialic acid. This situation may not occur *in vivo*, since FSH is always synthesized as a mixture of glycosylation variants whose relative proportion is determined by the hormonal milieu.

Nevertheless, as described by Ambao *et al*. (2009) 18% of total recovered pituitary FSH charge analogues present in immature male rats were isolated at the highest extreme of the pH gradient.

When the effect of rhFSH oligosaccharide complexity was analysed on inhibin B production, no consistent results were obtained. However, a previous study demonstrated that the less acidic/sialylated rhFSH charge analogue preparation contains a predominant proportion of rhFSH glycosylation variants bearing incomplete oligosaccharides (Loreti *et al*., 2013a). Therefore, it cannot ruled out that these glycoforms may contribute to stimulate inhibin B production in the immature Sertoli cell.

The high capacity to produce inhibin B that was observed at this stage of cell maturation led us to explore the existence of gonadal factors that may participate in the maintenance of this production.

Previous studies showed that activin A was a potent *stimulus* for inhibin B production in rat granulosa cells (Lanuza *et al*., 1999). Thus, the existence of a similar effect in the male gonad was explored. Our results confirmed the stimulatory effect of activin A on the production of inhibin B in Sertoli cells isolated from 8-day-old rat testes.

Peritubular cells produce activin A and the expression of its receptors has been described in immature rat Sertoli cells (Buzzard *et al*., 2004; Fragale *et al*., 2001). A classically described method to isolate immature Sertoli cells was used in this study; therefore, peritubular cells may have been present in the culture. We evaluated the possibility that locally produced activin A was responsible, at least in part, for the high inhibin B production in immature Sertoli cells. To evaluate this possibility an additional treatment with a hyperosmotic solution of glycine during the isolation process was

performed in order to reduce the number of peritubular cells present in the culture (Lejeune *et al*., 1993). The importance of activin A action was clearly demonstrated by the abrupt decrease in inhibin B production observed under these experimental conditions.

It is interesting to note that FSH and activin A synergistically stimulated inhibin α-subunit production; nevertheless, this gonadotrophin did not amplify activin A action on inhibin B levels. These results confirm the hypothesis that the expression of the inhibin/activin βB-subunit may be limiting the formation of the heterodimer at this stage of Sertoli cell maturation.

Less sialylated rhFSH charge analogues (BA) and those glycosylation variants with incomplete oligosaccharides (HY) were the most potent preparations to stimulate inhibin production in a more advanced stage of Sertoli cell maturation. This differential effect was observed both in the production of free inhibin α-subunit and inhibin B. These cells showed a higher capacity to respond to native rhFSH and its glycosylation variants in terms of free inhibin α-subunit production; thus maintaining the abundance of this peptide to enable inhibin B synthesis.

Molecular mechanisms involved in these differential responses have not been identified yet. It has been proposed that FSH glycosylation variants may have the capacity to activate different signal transduction pathways (Padmanabhan *et al*., 1991; Zambrano *et al*. 1996; 1999; Arey *et al*., 1997). Several studies have demonstrated that apart from the canonical Gs/cAMP/PKA pathway described for FSH, other alternative signalling pathways are involved in the mechanism of action of this gonadotrophin (Walker & Cheng, 2005; Gloaguen *et al*., 2011). More recent studies have proposed

that FSH oligosaccharide structure affecting the hormone-receptor complex conformation would allow the activation of different signal transduction pathways, thus inducing different biological responses (Ulloa-Aguirre *et al*., 1999; 2003; 2011; Landomiel *et al*., 2014).

Considering the importance of the action exerted by factors produced in the seminiferous epithelium on inhibin production at this advanced stage of Sertoli cell maturation, the differential effect of FSH glycosylation variants was evaluated in the presence of such factors. Under these experimental conditions, the rhFSH sialylation degree was unable to differentially modulate the inhibin α-subunit and inhibin B production. Conversely, the complexity of rhFSH oligosaccharide maintained its action on the regulation of these peptides production even in the presence of gonadal factors. These results suggest that the complexity of FSH oligosaccharides has a higher hierarchy than the sialylation degree in the differential effect on monomeric and dimeric inhibin production.

Different mechanisms modulate inhibin production in the Sertoli cell depending on its maturation stage. FSH appears to be an essential stimulatory factor to maintain inhibin α-subunit synthesis; however, peritubular cell-derived activin A may play a major role in sustaining immature Sertoli cell inhibin B production. The action of FSH and the presence of germ cells in the seminiferous tubules seem to be determinant for the heterodimer synthesis in Sertoli cells at a more advanced stage of maturation.

The findings obtained in the present study demonstrate that FSH glycosylation variants participate in the regulatory mechanisms of inhibin production and interact with factors produced by testicular cells at different stages of Sertoli cell maturation.

Declaration of interest

The authors declare that there is no conflict of interest that could prejudice the impartiality of the present research reported.

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Figure Legends

Figure 1. Effect of native rhFSH and its glycosylation variants on inhibin production by immature Sertoli cells. Cells isolated from 8-day-old rats were cultured in the absence or presence of increasing doses of native rhFSH (0.08 - 5 ng/mL, panels A and B) or the specified concentrations of rhFSH glycosylation variants. rhFSH-AC: more acidic/sialylated charge analogues, rhFSH-BA: less acidic/sialylated charge analogues (panels C and D). Doses are expressed in terms of hFSH-2 standard (NIDDKNHPP). Inhibin B (panels A and C), inhibin α-subunit (Pro-αC, panels A and D) and oestradiol (panel B) levels were determined in the conditioned media after 24h of treatment. Cyclic AMP levels (panel B) were determined in the conditioned media after 90 minutes. Data are expressed as mean ± SEM of triplicate determinations from one representative out of three independent experiments (panels A and B) or results are presented as fold increase over basal production and data expressed as mean ± SEM of three independent experiments, each performed in triplicate (panels C and D). (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$ compared with respective basal; (δ) $p < 0.05$ between the indicated experimental conditions.

Figure 2. Paracrine regulation of inhibin B and inhibin α-subunit production by immature Sertoli cells. Cells isolated from 8-day-old rats were treated with activin A (Act A, 50 ng/mL) or Follistatin (Fst, 100 ng/mL) (panels A and B). Sertoli cells isolated with ($S_{8\text{-gly}}$) or without (S_8) an additional glycine-EDTA treatment to remove peritubular cells, were cultured in the presence or absence of Act A (50 ng/mL) (panels C and E). Levels of inhibin B (panels A and C), inhibin α-subunit (Pro-αC, panels B and D) and Act

A (panels E and F) were determined in the conditioned media after 24h of treatment. Act A and Inh B levels were determined in the conditioned media of peritubular cells isolated from 8-day-old rat seminiferous tubule fragments after 24h of culture (panel F). Results are presented as fold increase over basal production and data expressed as mean ± SEM of three independent experiments, each performed in triplicate (panels A-E) or data are expressed as mean ± SEM of triplicate determinations from one representative out of three independent experiments (panel F). (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$ compared with respective control. ND: non detectable.

Figure 3. Effect of activin A and native rhFSH on inhibin production by immature Sertoli cells. Cells isolated from 8-day-old rats were cultured in the presence or absence of activin A (Act A, 50 ng/mL), native rhFSH (10 ng/mL) or the combination of both hormones. Inhibin B (panel A) and inhibin α-subunit (Pro-αC, panel B) levels were determined in the conditioned media after 24h of treatment. Results are presented as fold increase over basal production and data expressed as mean ± SEM of three independent experiments, each performed in triplicate. Different letters indicate significant differences between groups ($p < 0.05$).

Figure 4. Effect of native rhFSH and its glycosylation variants on inhibin production by mature Sertoli cells. Cells isolated from 20-day-old rats were cultured in the absence or presence of increasing doses of native rhFSH (0.1-10 ng/mL, panel A) or the specified concentrations of rhFSH glycosylation variants. rhFSH-AC: more acidic/sialylated charge analogues, rhFSH-BA: less acidic/sialylated charge analogues (panels B and C); rhFSH-CO: glycoforms bearing complex (triantennary and bisecting), rhFSH-HY: glycoforms bearing high mannose and hybrid-type oligosaccharides (panels

D and E). Inhibin B (panels A, B and D) and inhibin α-subunit (Pro-αC, panels A, C and E) levels were determined in the conditioned media after 72h of treatment. Data are expressed as mean ± SEM of triplicate determinations from one representative out of three independent experiments (panel A) or results are presented as fold increase over basal production and data expressed as mean ± SEM of three independent experiments, each performed in triplicate (panels B-E). (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$ compared with respective basal; (δ) $p < 0.05$ between the indicated experimental conditions.

Figure 5. Effect of native rhFSH, its glycosylation variants and gonadal factors on inhibin production by mature Sertoli cells. Cells isolated from 20-day-old rats were cultured in the presence or absence of TGF-β (1 ng/mL); EGF (50 ng/mL); Insulin (10 µg/mL); and native rhFSH or different rhFSH glycoforms (10 ng/mL) as indicated; rhFSH-AC: more acidic/sialylated charge analogues, rhFSH-BA: less acidic/sialylated charge analogues (panels C and D); rhFSH-CO: glycoforms bearing complex (triantennary and bisecting), rhFSH-HY: glycoforms bearing high mannose and hybrid-type oligosaccharides (panels E and F). Inhibin B (panels A, C and E) and inhibin α-subunit (Pro-αC; panels B, D and F) levels were determined in the conditioned media after 72 h of treatment. Results are presented as fold increase over basal production and data expressed as mean ± SEM of three independent experiments, each performed in triplicate. Different letters indicate significant differences between groups ($p < 0.05$). (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$ between the indicated experimental conditions.

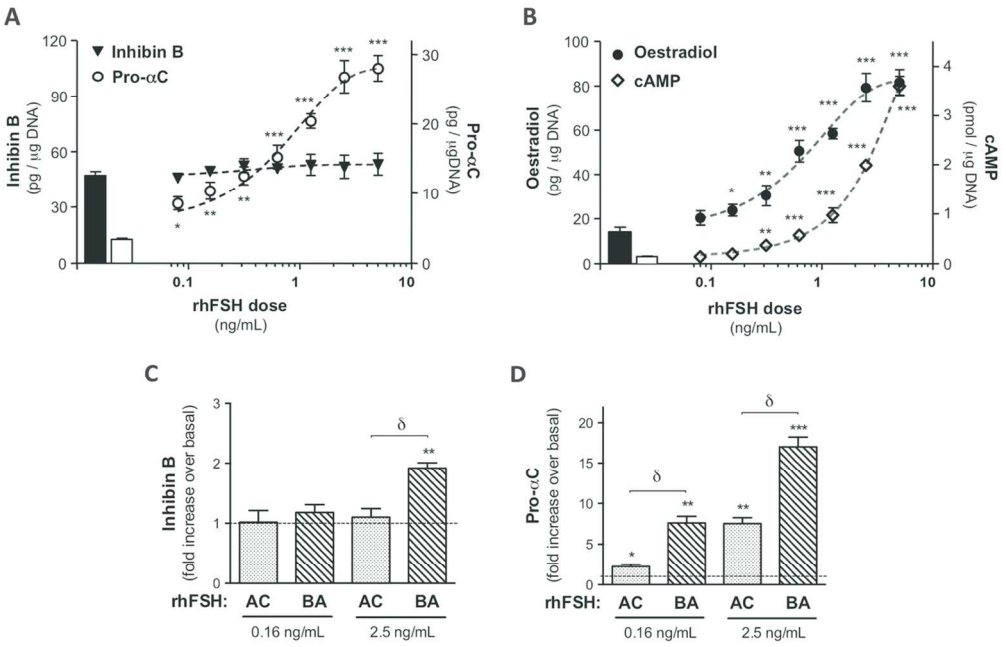

Figure 1. Effect of native rhFSH and its glycosylation variants on inhibin production by immature Sertoli cells.

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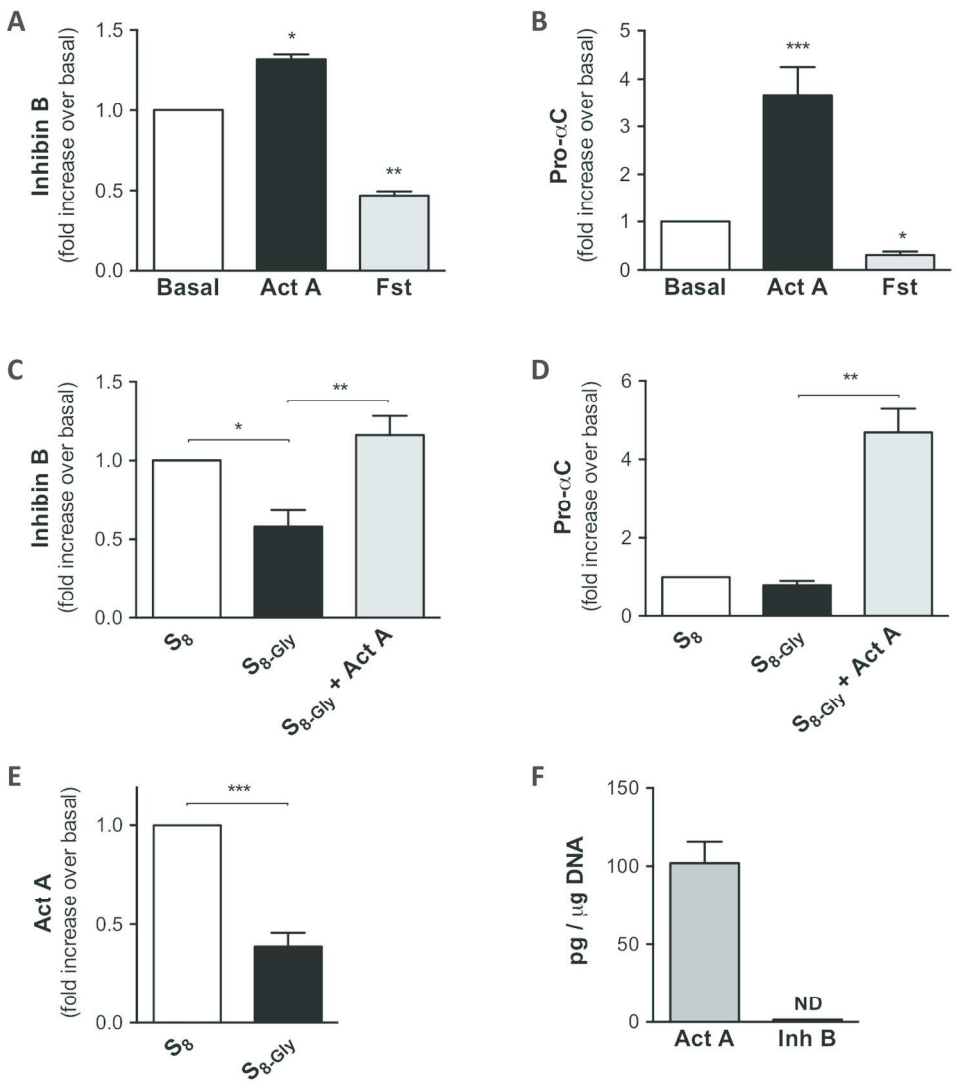

Figure 2. Paracrine regulation of inhibin B and inhibin α-subunit production by immature Sertoli cells.

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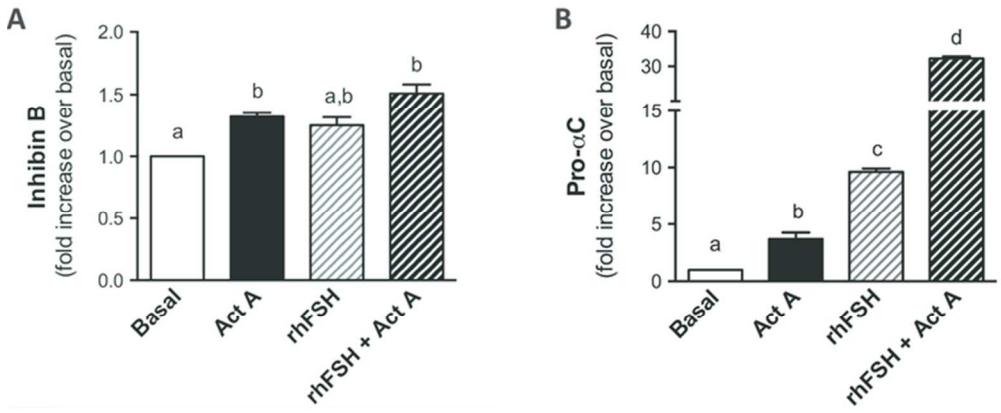

Figure 3. Effect of activin A and native rhFSH on inhibin production by immature Sertoli cells.

64x26mm (300 x 300 DPI)

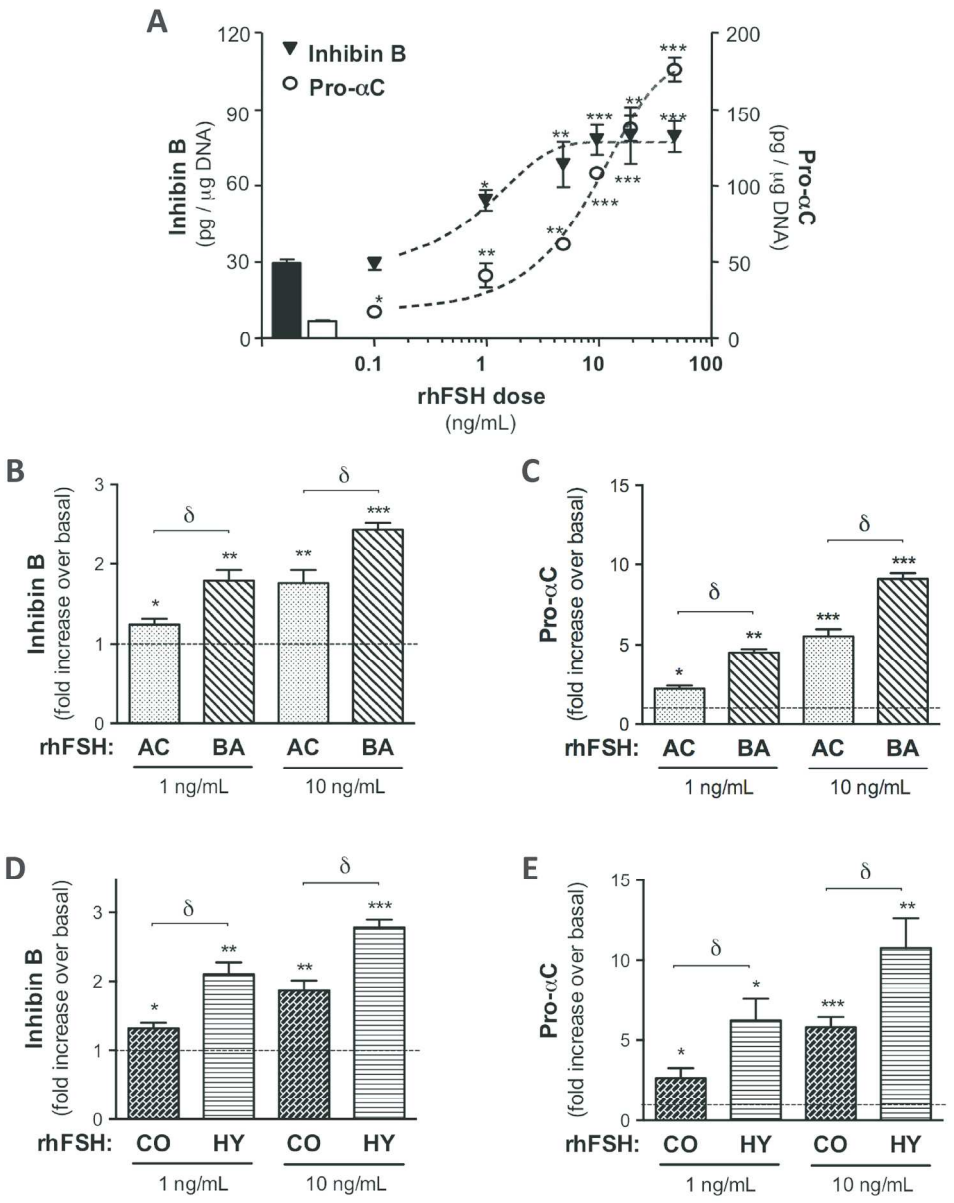

Figure 4. Effect of native rhFSH and its glycosylation variants on inhibin production by mature Sertoli cells.

166x203mm (300 x 300 DPI)

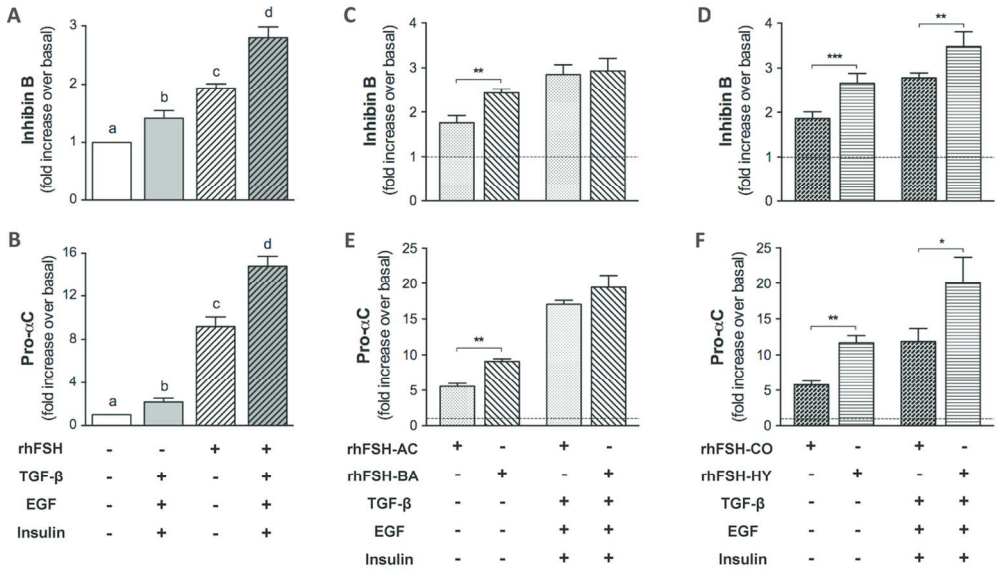

Figure 5. Effect of native rhFSH, its glycosylation variants and gonadal factors on inhibin production by mature Sertoli cells.

122x70mm (300 x 300 DPI)