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

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Abstract

Variations in follicle-stimulating hormone (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 SC. 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.

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Introduction

The Sertoli cell (SC) 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 SC function; supports cell proliferation before puberty, participates in cell maturation and regulates the synthesis of SC-derived products, which are essential for germ cell survival and function in the adult testis (Meachem et al. 2005).

Similar to other glycoprotein hormones, FSH consists of a family of glycosylation variants that 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 (Ulloa-Aguirre et al. 1986, Padmanabhan et al. 1988a,b, Wide 1989, Simoni et al. 1992, Creus et al. 1996, 2001). 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 amenorrhea 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 SC endocrine activity has never been explored.

During the last years, inhibin B (INHB) and anti-Müllerian hormone (AMH) have been considered reliable markers of SC function (Lah lou & 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, INHB 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 SCs have a great capacity to produce INHB independently of FSH stimulus and in the absence of spermatogenesis (Bergadá et al. 1999, Bergadá et al. 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 SC 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 (Bethesda, MD, USA). Insulin, TGF-β1, EGF, rat IL1-β and transferrin were purchased from Sigma-Aldrich. Tissue culture media were obtained from Gibco by Thermo Fisher Corporation. 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 killed by asphyxiation with CO2 and decapitation or cervical dislocation respectively, and the testes were removed for SC 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

SCs 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, 0.003% soybean trypsin inhibitor and 0.03% DNAse. The SC suspension, collected by sedimentation, was resuspended in the culture medium described above with the following additions: 10 μg/mL transferrin, 5 μg/mL insulin, 5 μg/mL vitamin E and 4 mg/mL hydrocortisone. SCs were cultured in 24-multiwell plates (15 μg DNA/cm2) at 34°C in a mixture of 5% CO2:95% air. The proportion of peritubular myoid cells present in the immature SC cultures was in the range of 10–15%, as evaluated by α-smooth muscle actin (α-SMA) immunostaining. When the extra glycine-EDTA treatment was applied during the cell isolation procedure, this proportion decreased to 3–5%.

SCs from 20-day-old Sprague–Dawley rats were isolated as previously described (Meroni et al. 1999, 2002). Briefly, decapsulated testes were digested with 0.1% collagenase and 0.006% soybean trypsin inhibitor in Hank’s balanced salt solution for 5 min at room temperature. Seminiferous tubules were saved, cut and submitted to 1 M glycine–2 mM EDTA (pH 7.4) treatment to remove peritubular cells. The washed tubular pellet was then digested again with collagenase for 10 min at room temperature to remove germinal cells. The SC suspension, collected by sedimentation, was resuspended in culture medium which consisted of a 1:1 mixture of Ham’s F-12 and DMEM, supplemented with 20 mM HEPES, 100 IU/mL penicillin, 2.5 μg/mL amphotericin B, 1.2 mg/mL sodium bicarbonate, 10 μg/mL transferrin, 5 μg/mL insulin, 5 μg/mL vitamin E and 4 mg/mL hydrocortisone. SCs were cultured in 24-multiwell plates (5 μg DNA/cm2) at 34°C in a mixture of 5% CO2:95% air. No peritubular myoid cell contamination was revealed in the cultures when an immunoperoxidase technique was applied to SC 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.

SCs 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 for 24 h (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 SCs were incubated in the presence of 0.1 mM IBMX and media were collected after 90 min of treatment. To evaluate aromatase activity, 2 mM 19-hydroxyandrostenedione as substrate for aromatization was added on day 4.

Following incubation, SCs 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 and Paigen (1980).

Peritubular cell isolation and culture

To isolate peritubular cells, seminiferous tubule fragments from 8-day-old rats, obtained after the initial dispersion, were seeded at low density (5 μg DNA/cm2) in the presence of 10%
FCS. After 7 days, the peritubular cell monolayer was collected by trypsin/EDTA treatment and seeded in 12-multwell 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) 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) as described previously (Bedecarras et al. 1998, Loreti et al. 2009, 2013a,b). Focusing was carried out at 12 W constant power (Power Pac 3000, Bio-Rad Laboratories) for 4 h, 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 dialyzed against 1 M NaCl to completely eliminate amphotelytes and detergent and 1-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 2.5 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 and coworkers (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 MgCl₂, 1 mM CaCl₂, and 1 mM MnCl₂) 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 dialyzed 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 SC function. They were selected based on the previously observed variations in the relative proportion of these two types of glycosylation variants when determined throughout sexual development in boys and male rats (Rulli et al. 1999, Campo et al. 2007).

**Hormone measurements**

The rhFSH content of samples was measured using an in-house double-antibody RIA with reagents provided by NIDDK (Bethesda, MD, USA). The RP LER-907 (1 mg LER-907Z 531U Second International Reference Preparation, hMG) was used to construct the standard curve. The polyclonal antibody, anti-hFSH-6, was used as antiserum. Purified hFSH (hFSH-ISIAPF-1) was iodinated using the chloramine-T method (Greenwood et al. 1963). To minimize the effects of interassay variations, as well as to determine the degree of parallelism between the unknown samples and the FSH standards, all glycoform preparations were analyzed at multiple dose levels in the same assay run. The intra- and interassay coefficients of variation were <9% and 12% respectively. Simultaneous curve fitting of the dose–response curves obtained in the RIA of the glycoforms revealed no significant differences among the slopes generated by the standard LER-907 and the different rhFSH glycosylation variants, suggesting that the glycoforms were equally recognized by the antibody. rhFSH is expressed in terms of hFSH-2 standard (NIDDKHPP).

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

Dimeric inhibins A and B, free inhibin α-subunit (Pro-αC) and total activin A levels in the culture media were measured using specific two-site enzyme-linked immunosorbent assays (ELISA) (Oxford Bio-Innovation Ltd, Oxon, UK) as described previously (Groome et al. 1994, 1995, 1996, Knight et al. 1996). Recombinant human inhibin A and B and activin A (Genentech, San Francisco, CA, USA), and a partially 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 INHB had less than 0.1% cross-reaction in dimeric inhibin assays. The Pro-αC assay had less than 0.1% cross-reactivity with inhibin A, B, activin A 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).

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Determination of cyclic AMP production

Immature SCs were cultured for 90 min in the presence of 0.1 mM 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, Paris, France). 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 were expressed as mean ± s.e.m. 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 *P < 0.05. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, www.graphpad.com).

Results

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

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

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

Inhibin A was always undetectable in SC cultures.

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

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

Native rhFSH did not induce changes in basal INHB production at any of the doses used in this study (Fig. 1, panel A). The production of cAMP and oestradiol was evaluated to confirm SC 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 ng/mL (1.53 ± 0.17-fold increase over basal, *P < 0.05). When 2.5 ng/mL 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).

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) 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 rhFSH-2 standard (NIDDK/NHP). 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 24 h of treatment. Cyclic AMP levels (panel B) were determined in the conditioned media after 90 min. Data are expressed as mean ± s.e.m. 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 ± s.e.m. 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; #P < 0.05 between the indicated experimental conditions.
Based on these results, the selected doses of rhFSH glycosylation variants chosen to assess possible differential biological effects on SCs were 0.16 ng/mL 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 SCs. 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 SC cultures.

No changes were observed in INHB production when the lowest dose of the two charge analogue preparations was used; whereas at the dose of 2.5 ng/mL INHB 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 INHB 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 SCs to produce considerable amounts of INHB under basal conditions after 24 h 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 INHB 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 to 53% and 69% respectively (P < 0.05) (Fig. 2, panels A and B).

An additional step in the isolation process to significantly reduce the presence of peritubular cells in the culture was performed. Under these experimental conditions a significant decrease in INHB production was observed (42%, P < 0.05) (Fig. 2, panel C). Concomitantly, the SC culture depleted of peritubular cells (S8-Gly) showed a 61% decrease in the production of activin A (P < 0.001, Fig. 2, panel E). The response of S8-Gly cells to the addition of exogenous activin A was as expected; INHB production was markedly stimulated: 1.61 ± 0.07-fold increase over S8-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 S8-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 INHB (Fig. 2, panel F).

The effect of combined activin A and rhFSH on INHB production was evaluated to recreate the in

![Figure 2](https://www.reproduction-online.org/2017/154/711-721/fsh-oligosaccharides-and-inhibin-production.png)
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-\( \alpha \)-C 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 SCs isolated from 20-day-old rats**

Increasing doses of rhFSH (0.1–50 ng/mL) were used to assess its effect on SC inhibin production. Pro-\( \alpha \)-C 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 INHB 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 INHB production 2.76 ± 0.21-fold over basal (\( P < 0.001 \)); higher doses of the gonadotrophin 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 ng/mL and 10 ng/mL), on 20-day-old rat SCs 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 INHB and Pro-\( \alpha \)-C production more markedly than the more acidic ones at both doses used (1 ng/mL and 10 ng/mL): INHB, 1.79 ± 0.13 vs 1.24 ± 0.07 and 2.43 ± 0.09 vs 1.76 ± 0.16-fold increase over basal; Pro-\( \alpha \)-C: 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 INHB and Pro-\( \alpha \)-C production at 1 ng/mL and 10 ng/mL (\( P < 0.05 \), Fig. 4, panels D and E). Glycoforms bearing high mannose and hybrid-type oligosaccharides stimulated

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**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. INHB (panel A) and inhibin \( \alpha \)-subunit (Pro-\( \alpha \)-C, panel B) levels were determined in the conditioned media after 24 h of treatment. Results are presented as fold increase over basal production and data expressed as mean ± S.E.M. 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). INHB (panels A, B and D) and inhibin \( \alpha \)-subunit (Pro-\( \alpha \)-C, panels A, C and E) levels were determined in the conditioned media after 72 h of treatment. Data are expressed as mean ± S.E.M. 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 ± S.E.M. of three independent experiments, each performed in triplicate (panels B, C, D and E). *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \) compared with respective basal; \( P < 0.05 \) between the indicated experimental conditions.
INHB and Pro-αC production more effectively than those bearing complex oligosaccharides: INHB: 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 rFSH and its glycosylation variants.

When the effect of combined TGF-β, EGF and insulin was evaluated in INHB, 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 rFSH on INHB 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 rFSH 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 rFSH was evaluated a 32% reduction of the basal production was observed (P<0.05).

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

When the effect of rFSH sialylation degree on INHB and Pro-αC production was evaluated in the presence of combined TGF-β, EGF and insulin, both rFSH charge analogues preparations exerted a similar stimulation (2.85 ± 0.22 vs 2.93 ± 0.28 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 rFSH oligosaccharides on INHB and Pro-αC production was maintained even in the presence of gonadal factors (2.66 ± 0.22 vs 3.48 ± 0.33 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,b).

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

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**Table 1:**

<table>
<thead>
<tr>
<th>Condition</th>
<th>INHB (fold increase)</th>
<th>Pro-αC (fold increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2.11 ± 0.17</td>
<td>6.21 ± 1.34</td>
</tr>
<tr>
<td>rFSH</td>
<td>2.80 ± 0.18</td>
<td>14.81 ± 0.90</td>
</tr>
<tr>
<td>TGF-β, EGF, Insulin</td>
<td>2.85 ± 0.22</td>
<td>17.06 ± 0.54</td>
</tr>
<tr>
<td>rFSH-AC</td>
<td>2.93 ± 0.28</td>
<td>20.04 ± 3.64</td>
</tr>
<tr>
<td>rFSH-BA</td>
<td>2.66 ± 0.22</td>
<td>3.48 ± 0.33</td>
</tr>
<tr>
<td>rFSH-HY</td>
<td>11.68 ± 1.01</td>
<td>20.04 ± 3.64</td>
</tr>
</tbody>
</table>

*Note: All values are mean ± SEM of three independent experiments, each performed in triplicate. Different letters indicate significant differences between groups (P<0.05).*
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 SC maturation. Cultured immature SCs 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 SC 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 SCs 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 SCs in culture were the high capacity of these cells to produce INHB 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 inhibit α-subunit in a dose-dependent manner.

Based on the abovementioned results regarding the high oestradiol production induced by hFSH charge analogues isolated at pH>5 on immature SCs, we have 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 INHB production, since native rhFSH did not change basal levels.

These results indicate that the important INHB 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 and coworkers (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 analyzed on INHB 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 be ruled out that these glycoforms may contribute to stimulate INHB production in the immature SC.

The high capacity to produce INHB 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 INHB 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 INHB in SCs isolated from 8-day-old rat testes.

Peritubular cells produce activin A and the expression of its receptors has been described in immature rat SCs (Fragale et al. 2001, Buzzard et al. 2004). A classically described method to isolate immature SCs 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 INHB production in immature SCs. To evaluate this possibility an additional treatment with a hyperosmotic solution of glycine during the isolation process was performed 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 INHB 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 INHB levels. These results confirm the hypothesis that the expression of the inhibin/activin β B-subunit may be limiting the formation of the heterodimer at this stage of SC 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 SC maturation. This differential effect was observed both in the production of free inhibin α-subunit and INHB. 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 INHB 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, Arey et al. 1997, 1999). Several studies have demonstrated that apart from the canonical Gs/cAMP/ PKA pathway described for FSH, other alternative signaling pathways are involved in the mechanism of action of this gonadotrophin (Walker & Cheng 2005, Gloaguenn 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.

Considering the importance of the action exerted by factors produced in the seminiferous epithelium on inhibin production at this advanced stage of SC 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 INHB 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 SCs 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 SC INHB production. The action of FSH and the presence of germ cells in the seminiferous tubules seem to be determinant for the heterodimer synthesis in SCs 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 SC 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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