

Concanavalin-A Induces Granulosa Cell Death and Inhibits FSH-Mediated Follicular Growth and Ovarian Maturation in Female Rats

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Reproductive success stems from a finely regulated balance between follicular maturation and atresia, in which the role of carbohydrate structure is poorly understood. Here, we describe for the first time a fraction of purified recombinant human FSH that is capable of bringing about the cell death of granulosa cells and preventing follicular maturation in a rat model. Further analysis by mass spectrometry revealed the presence of the lectin Concanavalin-A (Con-A) within this fraction of recombinant FSH. Using both the fractionated FSH and Con-A, the observed cell death was predominantly located to the granulosa cells. Ex vivo culture of rat follicles demonstrated that follicle degeneration occurred and resulted in the release of a denuded and deteriorated oocyte. Moreover, in vivo experiments confirmed an increase in atresia and a corresponding reduction confined to follicle in early antral stage. As a mechanism of action, Con-A reduces ovarian proliferation, Von Willebrand staining, and angiogenesis. Based on the observation that Con-A may induce granulosa cell death followed by follicle death, our results further demonstrate that follicular carbohydrate moiety is changing under the influence of FSH, which may allow a carbohydrate-binding lectin to increase granulosa cell death. The physiological consequences of circulating lectin-like molecules remain to be determined. However, our results suggest a potential exploitation of carbohydrate binding in fertility and ovarian cancer treatment. This work may shed light on a key role of carbohydrates in the still obscure physiological process of follicular selection and atresia. (*Endocrinology* 154: 1885–1896, 2013)

The ovarian follicle is considered the functional unit of the ovary. Every follicle contains a female germ cell, an oocyte (or egg), and the primary role of the follicle is to provide the necessary support to enable the development and maturation of the oocyte (1). Early follicular development and endocrine activity of antral follicles in their

progress towards a preovulatory state (and therefore oocyte maturation) is dependent on FSH, which promotes survival, proliferation, steroidogenic activity, and differentiation of granulosa cells (2–5). Currently, recombinant human (rh)FSH is used for the practice of assisted reproductive technologies, including ovarian stimulation ther-

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Abbreviations: Con-A, Concanavalin-A; DES, diethylstilbestrol; IEF, isoelectric focusing; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt; NIH, National Institutes of Health; PCNA, proliferating cell nuclear antigen; rh, recombinant human; TUNEL, terminal deoxynucleotidyl transferase 2'-deoxyuridine, 5'-triphosphate nick end labeling.

apy, anovulation disorders, polycystic ovary syndrome, and induction of spermatogenesis in hypogonadotropic males (6–8).

FSH is secreted into circulation as glycosylated variants due to molecular heterogeneity in the composition and inner structure of carbohydrate chains (9, 10). Although the physiological role of FSH glycosylation has not been well established, it is recognized that oligosaccharides are required for biological activity (11). FSH molecular variant profile has been established in human and mammalian species during different physiological stages (12–15), and changes are described during transition through male puberty (16), in postmenopause (17), during lactational amenorrhea (18), and during the menstrual cycle (12, 14, 19, 20). In each menstrual cycle, it selects the dominant follicle, whereas remaining maturing follicles become atretic. Although still not fully understood, mechanisms of atresia are mainly ascribed to classical intrinsic and extrinsic apoptotic cell death pathways, there is also evidence for caspase-independent mechanisms (21–23).

Lectins are widely used in the purification of enzyme-antibody conjugates, the isolation of cell surface glycoproteins, and in recombinant hormone isolation, due to their carbohydrate binding capacity (8). The lectin Concanavalin-A (Con-A) is a tetrameric metalloprotein isolated from the Jack bean, which binds to α -D-mannopyranosyl, α -D-glucopyranosyl, and sterically related residues with high affinity (24). Con-A coupled to sepharose is commonly used for enrichment, separation, and purification of glycoproteins, polysaccharides, and glycolipids. Recently, Con-A and other lectins have been speculated to possess proapoptotic, proautophagic, and antiangiogenic properties in cancer cell lines (reviewed in Refs. 25, 26).

Here, the aim of this study was to demonstrate that the changes in carbohydrate moiety of a human rhFSH could bring about changes in biological activity of maturing rat follicles. As this article will recount, a notable and unexpected change in biological activity was observed, but this activity upon the maturing rat ovary was under the control of the lectin Con-A, inadvertently concentrated from the recombinant protein preparation. However, the manner and specificity of the observed granulosa cell death suggest a role for glycosylation in physiological follicular atresia.

Materials and Methods

Animals

Immature Sprague-Dawley female rats (21–23 d old) were obtained from the Pontificia Universidad Católica de Chile, and protocols were approved by the bioethics committee. Animals

were kept under a controlled environment (temperature 21°C–24°C and 12-hour light, 12-hour dark cycles, food and water ad libitum). Animal killing was performed by cervical dislocation. All protocols were approved by the Bioethics and Biosafety Committee of the School of Biological Sciences, Pontificia Universidad Católica de Chile.

Reagents

Chemicals were of analytical grade from Merck S.A. (Santiago, Chile). Androstenedione, HEPES, BSA, insulin, transferrin, L-glutamine, ascorbic acid, diethylstilbestrol (DES), Con-A type VI, rabbit polyclonal anti-*Canavalia ensiformis* lectin (Con-A) antibody and Con-A peroxidase conjugate, and methyl- α -D-mannopyranoside were purchased from Sigma-Aldrich, Inc (St. Louis, Missouri). Con-A sepharose was purchased from Amersham (Piscataway, New Jersey). GIBCO DMEM:F12 (4.5-g glucose/L) media were from Invitrogen (Grand Island, New York). rhFSH and all reagents for FSH quantification were purchased from Dr A. F. Parlow at the National Hormone and Peptide Program of the National Institute of Diabetes and Digestive and Kidney Diseases-National Institutes of Health (NIH) (Torrance, California). Antirabbit IgG and normal serum rabbit were from Peninsula Labs, Bachem Group (San Francisco, California). Rabbit polyclonal anti-Von Willebrand factor from DAKO (Glostrup, Denmark), rabbit polyclonal proliferating cell nuclear antigen (PCNA) from Santa Cruz Biotechnology, Inc (Santa Cruz, California), and Vectastain ABC system from Vector Laboratories (Burlingame, California) were used to immunohistochemical analysis. Antirabbit secondary antibody horseradish peroxidase conjugated from Bio-Rad Labs (California) and Western Lightning ECL system from PerkinElmer (Waltham, Massachusetts) were used for Western blot analysis. Ketamine was purchased from Holliday-Scott (Buenos Aires, Argentina), and xylazine was obtained from König Laboratories (Buenos Aires, Argentina). For specific information of antibodies used in this study, please see Supplemental Table 1, published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>.

Isolation of FSH fractions

FSH fractions from a rhFSH preparation (rhFSH) purchased from the National Institute of Diabetes and Digestive and Kidney Diseases-NIH were obtained through a 2-step fractionating procedure consisting of preparative isoelectric focusing (IEF) followed by lectin affinity chromatography, as previously described (18). Briefly, IEF was carried out as previously described (27) with particular focusing condition directed to get an extended pH range (1.5% CHAPS; 1.4% ampholytes pH range 2.5–5 and 3% ampholytes pH range 3–10). Pools from fractions obtained at pH less than 4.1 (pool A) or pH more than 4.5 (pool B) were individually applied into a Con-A-sepharose column. Lectin affinity chromatography was carried out as previously described (28) and modified (17), and 6 FSH fractions were obtained (fractions 1–3 from pool A, fractions 4–6 from pool B). FSH quantification was carried out using RIA (10). All FSH fractions were analyzed at multiple dose levels in the same assay run. The intra- and interassay coefficients of variation were less than 9% and 12%, respectively. The sensitivity of the assay was 4.5 ng (LER-907) per tube.

Western blot analysis

Five micrograms of rhFSH and FSH fractions obtained as previously described were mixed with sample buffer without 2-mercaptoethanol to avoid reducing protein to subunits. Some experiments were carried out in order to test changes on protein migration under heat denaturation. In that case, samples were boiled during 5 minutes to 95°C before to be loaded into the gel. Proteins were separated by 15% polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate and transferred to nitrocellulose membranes. The blot was preincubated in blocking buffer (5% nonfat milk, 0.05% Tween 20 in Tris-buffered saline 20mM [Tris:HCl 4mM and NaCl 100mM; pH 8]) for 1 hour at room temperature and incubated overnight with polyclonal antihuman FSH antibody (1:50 000) or polyclonal anti-Con-A antibody (1:50 000). Then, it was incubated with anti-rabbit secondary antibody coupled with horseradish peroxidase (1:3000) for 1 hour at room temperature. Bound antibody was detected by chemiluminescence using the Western Lightning ECL system.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Proteins from a silver stained gel were gel-in-gel digested by trypsin and analyzed by LC-MS/MS using Orbitrap Velos Mass Spectrometer at the Turku Centre for Biotechnology. Database searches were performed by Mascot against SwissProt (UniProt) protein sequence database.

Primary cultured rat granulosa cells

Ovarian granulosa cells were isolated from DES-treated rats as previously described (29). Diethylstilbestrol priming is a well-established experimental procedure to promote follicular development up to early antral follicle stage in immature rats (2, 29, 30). Granulosa cells were seeded onto 96- or 6-well plates (Nunc, Roskilde, Denmark) precoated with rat-tail collagen at initial plating density of 1×10^6 viable cells/mL. After 3 hours, non-attached cells were removed, and 24 hours later, treatments were added in fresh media containing 5- μ g/mL insulin and 25 μ M androstenedione. Previous data demonstrated that FSH at a concentration of 20 ng/mL significantly increased estradiol production in this system; therefore, this concentration of FSH was used in all experiments of granulosa cell culture.

Individual rat follicle culture

Ovaries from immature DES-treated rats were used to obtain early antral follicles. It has been previously reported that follicles with more than 6–7 layers of granulosa cells had initiated antral formation, in rodents this condition is observed since a size of 200 μ m (1, 31, 32). Early antral follicles (~250 μ m) were mechanically isolated by needle dissection as described (33) with some modifications. Follicles were collected in DMEM:F12 supplemented with 3-mg/mL BSA, 5- μ g/mL insulin, 1 μ M L-glutamine, 10- μ g/mL transferrin, 50- μ g/mL ascorbic acid, antibiotics, and with 10mM HEPES. For each experiment, a pool of follicles from 5–7 rats was selected according to size, clearness, granulosa homogeneity, and intact outer layer. Only follicles showing theca layer integrity and no visible damage during isolation were chosen for further culture. At least 6–7 healthy follicles were randomly assigned for each experimental condition. Follicles were transferred into 96-well plates and individually

cultured in DMEM:F12 supplemented with 3-mg/mL BSA, 5- μ g/mL insulin, 1 μ M L-glutamine, 10- μ g/mL transferrin, 50- μ g/mL ascorbic acid, antibiotics, 2.2-g/L NaHCO₃, and 25 μ M androstenedione. Every 24 hours, external diameter was measured, and follicles were transferred to a clean well with fresh medium containing corresponding treatment conditions. rhFSH or fractions were used at a concentration of 50 ng/mL and Con-A at 12.5 μ g/mL for follicle incubation. An additional group of follicles was cultured in the supplemented medium without FSH or Con-A (basal condition) as a control of the experiment. Follicle evaluation was performed using a Nikon stereomicroscope (Nikon, Melville, New York) as previously reported (33). After culture, follicles were fixed, dehydrated, and LR-White embedded (34), and 2- to 3- μ m slides were obtained for histological and terminal deoxynucleotidyl transferase 2'-deoxyuridine, 5'-triphosphate nick end labeling (TUNEL) analysis.

Because early follicle disruption may be associated to delayed signs of mechanical damage during the isolation procedure, follicles ruptured before 2 days in culture were excluded from the study. A total of 170 follicles was analyzed, each treatment condition group having at least 14 intact follicles at day 2.

In vivo experiments

Ovarian treatment by intrabursal administration of molecules was performed as previously described (35). Briefly, immature female Sprague-Dawley rats were anesthetized with ketamine HCl (80 mg/kg) and xylazine (4 mg/kg). The ovaries were exteriorized through an incision made in the dorsal lumbar region. In every case, one ovary was used as test condition, and the contralateral ovary was used as a control. In one group of animals (n = 9), rats received a combination of rhFSH and fraction 4 in one ovary. In other group (n = 6), animals received a combination of rhFSH and Con-A in one ovary. In both groups, the contralateral ovary was injected with the same volume of rhFSH, and it was used as a control. rhFSH or fraction 4 were used at a final concentration of 50 ng/mL and Con-A at 12.5 μ g/mL. In a third group of animals (n = 5), one ovary was treated with rhFSH, and in the contralateral side, a NaCl 10mM solution was used as control. After injection, ovaries were replaced and the incision closed with skin adhesive. Forty-eight hours later, the ovaries were removed, cleaned, fixed in 4% neutral-buffered formalin, and processed for paraffin inclusion.

Ovarian morphology and follicular classification

A classification modified from that of Pedersen and Peters (31) was used to define stages of follicular development. The first group defined was the unilaminar stage, which included primordial and primary follicles; preantral stage corresponded to follicles possessing 2–7 granulosa layers (<400 cells on the largest cross-section) and no follicular fluid; early antral stage corresponded to follicles with more than 5 granulosa layers, and it is characterized by the appearance of scattered areas of fluid; large antral stage was defined by follicles possessing a definite antral cavity. No other advanced stages of follicular development, such as preovulatory follicles or corpus luteus, were observed in the ovaries from DES-treated animals. It is noteworthy to mention that because early antral follicles have recently acquired small fluid areas, it is probable that certain cross-sections do not show the incipient cavity. However, all healthy follicles with more than

400 cells in the largest follicle were confirmed in serial cross-sections to possess antral formation.

Atretic follicles were classified into initial to mid atretic process (Atretic II) or late atretic stage (Atretic III) according to the criteria of Hsueh et al (36). Morphological characteristics of Atretic II include the presence of pyknotic nuclei in granulosa cells, degeneration and detachment of the granulosa layer from the basal membrane; at this stage, morphology of the follicle is still spherical or ovoid. In the advanced Atretic III process, follicle changes are characterized by loss of well-defined granulosa-theca layers, detachment of cumulus cells from the oocyte, partial or total elimination of granulosa cell layer, theca cell hypertrophy, collapse of the follicle structure, and oocyte degeneration (fragmentation or vacuolization). Due to the Atretic I stage corresponding to a very early process and possibly uniquely biochemical (without morphological changes), follicles in this stage may be unnoticed by simple observation with hematoxylin-eosin stain and therefore only follicles at stage II and III were counted. These criteria were applied for analysis of individually cultured follicles and whole ovaries from *in vivo* treatments.

Five-micrometer step ovarian sections for each sample were obtained from paraffin blocks. Follicular stage counting was performed on 5 hematoxylin-eosin-stained sections from each ovary, 6–9 ovaries per experimental condition. To prevent counting the same follicle twice, 5- μ m step sections were mounted at 50- μ m intervals onto microscope slides as previously reported (37).

Immunohistochemistry and lectin staining

Immunodetection of PCNA and Von Willebrand factor were carried out in 5- μ m step ovarian sections as previously described (38). Briefly, endogenous peroxidase activity was blocked with H₂O₂ solution, and nonspecific binding was blocked with 2% BSA-PBS overnight at 4°C. Sections were incubated with anti-Von Willebrand antibody (1:100) or anti-PCNA antibody (1:100) overnight at 4°C. After washing, the slides were incubated with biotinylated antirabbit IgG and revealed with avidin-biotinylated horseradish peroxidase complex. For the negative controls, primary antibody was omitted. The proliferation index (PCNA-positive cells expressed as a percentage of the total number of cells) was established in the granulosa compartment for each early antral follicle, 6 antral follicles for each ovary were counted. To determine the vascular area, endothelial cell density was established by the presence of Von Willebrand factor-positive cells as area occupied by blood vessels respect to the total area of the ovary. Six randomly selected fields were analyzed from each ovarian section, and a random counting procedure was used as previously described (38).

Lectin staining with Con-A peroxidase conjugate was performed with a direct method as previously described (39). Briefly, 5- μ m cross-section slides from ovaries treated with 10mM NaCl (control) or rhFSH were incubated during 30 minutes in 3% hydrogen peroxide to block endogenous peroxidase activity. After that, slides were incubated with Con-A lectin labeled with horseradish peroxidase (50 μ g/mL) for 24 hours at room temperature at 4°C. Ovarian areas positively stained for lectin binding were visualized with diaminobenzidine. All buffers were supplemented with salts MnCl₂, CaCl₂, and MgCl₂ at 1 mg/mL. Negative controls were preincubated for 1 hour at room temperature with 200mM methyl- α -D-mannopyranoside before lectin incubation.

Flow cytometry

Cell cycle distribution and detection of a sub-G₀/G₁ population was performed FACScan cytometer and the Cell Quest software (Becton Dickinson, California) as previously described (40).

Cell viability and DNA fragmentation analysis

Promega CellTiter 96 AQueous Cell Proliferation MTS Assay and DeadEnd Fluorometric TUNEL System from Promega (Madison, Wisconsin) were used according to manufacturer's instructions.

Data analysis

Images from immunohistochemical studies were analyzed using the NIH ImageJ software (Bethesda, Maryland). Statistical analysis was carried out using GraphPad Prism Software, Inc (San Diego, California). Comparison between multiple groups was by 1-way ANOVA followed by Tukey multiple comparison test. For pairwise comparisons, *t* test or the nonparametric Mann-Whitney analysis was applied. In follicle culture experiments, differences between groups in the proportion of follicles according to morphology (intact or spontaneous rupture) and histology (healthy or atretic) were analyzed by χ^2 test or Fisher's exact test as appropriate. Values of *P* < .05 were considered significant.

Results

An ultrapure preparation of rhFSH was fractionated using a 2-step separation procedure according to anticipated differences in FSH glycoforms. Firstly, proteins were separated by preparative IEF based on isoelectric point, and subsequently lectin affinity chromatography was used to obtain 6 pools of FSH (referred to hereafter as FSH fractions 1–6).

The principal actions of FSH are to stimulate proliferation and differentiation of granulosa cells and to promote follicular cell survival. To determine whether our fractions possessed differing biological activity, we cultured rat granulosa cells in the presence of rhFSH or each individual fraction. After 48 hours, cells were recovered and underwent cell cycle analysis by flow cytometry (Figure 1A). As estimated, rhFSH and 5 of the 6 FSH variants increased granulosa cell proliferation. However, unexpectedly, granulosa cells incubated with fraction 4 showed no increase but, instead, significantly decreased proliferation. Moreover, the proliferation, as measured by the cells in the S or G₂/M phase, was lower that of nontreated cells. Interestingly, fraction 4 caused an increase of the sub-G₀/G₁ peak, suggestive of cell death, whereas rhFSH and the other fractions reduced this percentage (Figure 1B). Confirming this observation by other techniques, fraction 4 but not fraction 1 (a fraction with different isoelectric point but the same lectin affinity characteristics), or rhFSH,

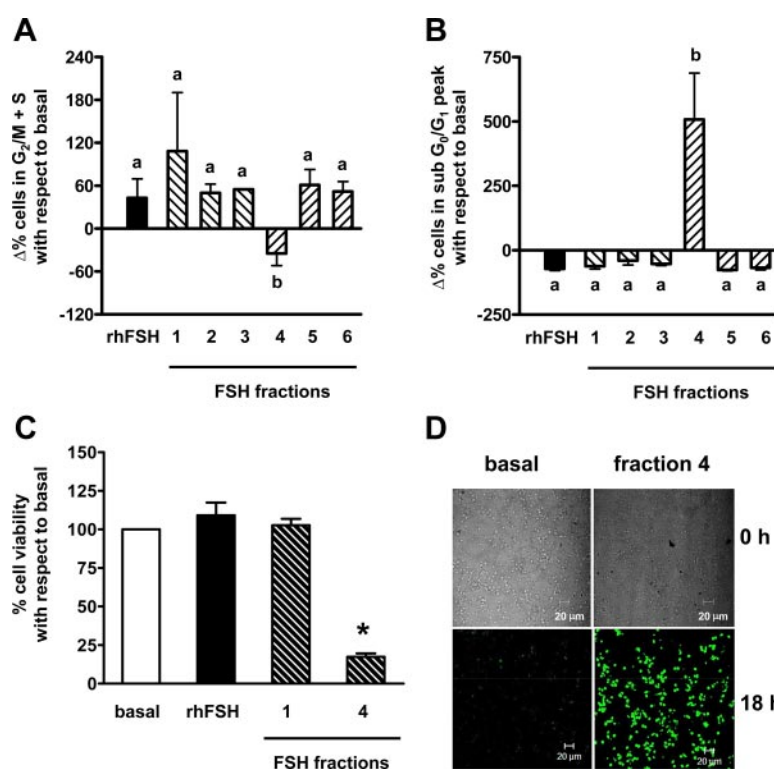


Figure 1. FSH-fraction 4 inhibits cell proliferation and increases cell death. Six FSH fractions were obtained from a preparation of rhFSH. The effects on cell cycle in rat granulosa cells were assayed for all FSH fractions by flow cytometry analysis (A and B) and MTS assay (C) after 48 and 24 hours of treatment, respectively. Primary cultures of granulosa cells were incubated in the presence of total rhFSH, FSH fractions, or basal conditions. As occurred with rhFSH exposure, most FSH fractions showed proliferative activity and protection from cell death. Uniquely, fraction 4 presented a significant decrease in cell viability at this time point. Analysis by TUNEL assay at 18 hours confirmed granulosa cell death in the presence of fraction 4 (D). Data are presented as mean \pm SE ($n = 3$). $a \neq b \neq$ basal condition, ANOVA $P < .05$; * ANOVA $P < .05$ in comparison with basal condition.

brought about a loss of cell viability by the 3-(4,5-dimethylthiazol-2-yl)5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay (Figure 1C). Analysis by the TUNEL assay (measuring DNA fragmen-

tation) after 18 hours of fraction 4 treatment confirmed the increase in cell death (Figure 1D).

Because the ovarian follicle is a functional unit that comprises auto-crine and paracrine factors being exchanged between different cell types, we evaluated the effect of fraction 4 on intact rat follicles. It is worth mentioning that this model has the advantage of maintaining the integrity of the follicle structure. Follicles at early antral stage were chosen due to their responsiveness and dependence on FSH to progress to maturation. Antral follicles ($\sim 250 \mu\text{m}$) were individually cultured in the presence of rhFSH or fractions 1 and 4. A summary of the results obtained from the ex vivo culture is given in Table 1. By the third day of culture, follicles incubated with rhFSH or fraction 1 had begun to demonstrate in vitro growth and manifested a lucent appearance. As confirmed by histological analysis, follicular cells were healthy (Figure 2A), follicles increased in diameter (Figure 2C), and possessed a low proportion of TUNEL-positive cells (Figure 2B). However, untreated follicles (basal) and follicles cultured with FSH-fraction 4 exhibited darkening and minimal growth (Figure 2, C and D). Histological analysis revealed degeneration of follicular structure, and TUNEL analysis demonstrated

Table 1. Effect of FSH-Fraction 4 and Con-A on Ex Vivo-Cultured Follicles

	Morphology		Histology		
	Intact (%)	Spontaneous rupture (%)	Healthy (%)	Atretic II (%)	Atretic III (%)
Effect of fraction 4					
Basal	93.1	6.9 ^a	6.9	82.8	10.3 ^a
rhFSH	37.9	62.1	86.2	10.3	3.4
Fraction 4	65.5	34.5 ^{ab}	3.2	32.3	64.5 ^{ab}
Fraction 1	45.8	54.2 ^b	87.5	12.5	0.0 ^b
Effect of Con-A					
Basal	93.3	6.7	20.0	73.3	6.7 ^a
rhFSH	35.7	64.3	78.6	21.4	0.0
Con-A	66.7	33.3	13.3	26.7	60.0 ^{ab}
rhFSH + Con-A	42.9	57.1 ^b	35.7	57.1	7.1 ^{ab}

^a $P < .05$, in comparison with group treated with rhFSH.

^b $P < .05$, in comparison with group in basal condition.

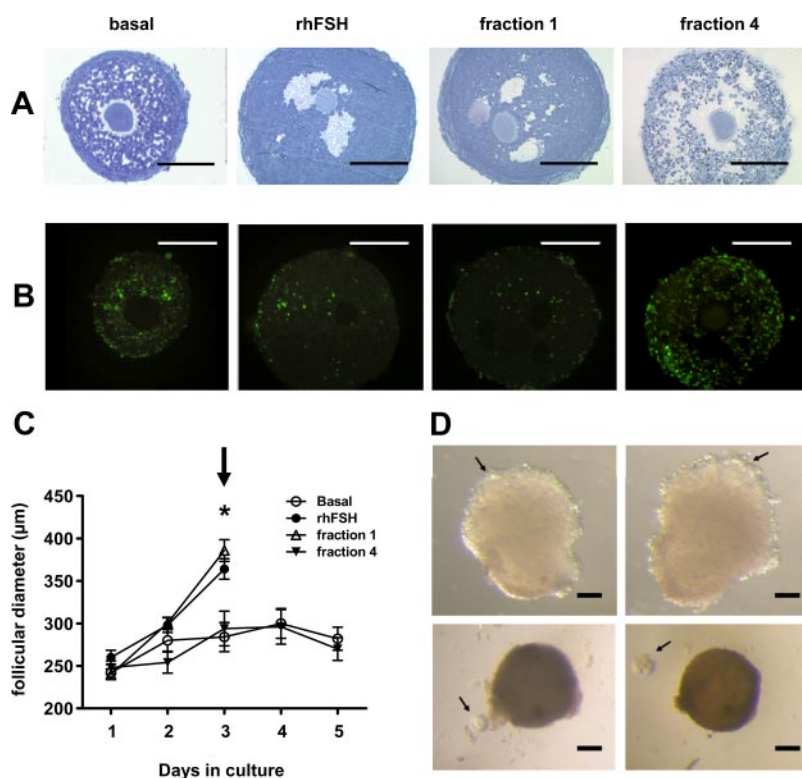


Figure 2. FSH-fraction 4 induces cell death in early antral follicles. Small antral follicles (~ 250 μm) were individually cultured in basal conditions or in the presence of FSH. Histological analysis after 3 days of treatment revealed that samples incubated with fraction 4 demonstrated severe degeneration of follicular structure (A) and more TUNEL-positive cells (B). In accordance with these observations, follicles incubated in the presence of fraction 4 presented limited-to-no growth (C) and demonstrated reduced size and a remarkably darkened appearance. When spontaneous rupture occurred, abnormal characteristics, such as cytolysis or fragmentation, were observed in the oocytes released from these follicles (D, lower panels). In contrast, follicles treated with fraction 1 (a proliferative and steroidogenic fraction of FSH shown previously to have similar properties to rhFSH) exhibited normal development and a healthy release of oocytes (D, upper panels). Representative images from each treatment are shown. Scale bar, 100 μm ; arrow indicates oocyte localization. Data are presented as mean \pm SE ($n = 3$). * ANOVA $P < .05$ in comparison with basal condition.

the presence of increased cell death in 4 treated follicles (Figure 2, A and B). Interestingly, after the third day of culture, most follicles incubated in the presence of fraction 1 or rhFSH had undergone spontaneous rupture (black arrow in Figure 2C and Table 1). These follicles showed an oocyte surrounded by numerous granulosa cells forming a brilliant expanded cumulus; the oocyte appearance was healthy, and no signs of degeneration were observed (Figure 2D, upper panels). Of the 62.1% of rhFSH-treated follicles that underwent spontaneous rupture, 94.4% of these demonstrated healthy histological characteristics. Conversely, of the 34.5% of fraction 4-treated follicles that underwent disruption, 100% had an atretic histology (Table 1). These latter oocytes had a total absence of follicular cells and showed liberation of a denuded oocyte (Figure 2D, lower panels). Moreover, the released oocyte appeared to have abnormal characteristics, suggesting cytolysis or fragmentation.

Given the unexpected activity of fraction 4, we wished to confirm the presence and nature of FSH in this fraction. Western blot analysis of fractions 1–6 demonstrated that FSH was present in each fraction in both native and denatured forms (~ 37 and 25 kDa, respectively), albeit varying slightly in migration (presumably due to glycosylation). However, we observed a unique band at approximately 26–30 kDa in fraction 4 (Figure 3A). Interestingly, unlike the protein bands reported for rhFSH, which migrated below 25 kDa upon heat denaturation, the migration of this unique band in fraction 4 did not alter upon denaturation, suggesting an unspecific antibody interaction and that the corresponding protein was not FSH (Figure 3B). To confirm this, we carried out electrophoretic analysis of fraction 4 and of rhFSH starting material and excised the unique band, along with the bands corresponding in size to FSH. The proteins were cut from a silver stained gel, in-gel digested by trypsin, and analyzed by LC-MS/MS. The protein sequence analysis confirmed FSH as the protein present in the expected bands. However, the analysis also revealed the presence of the lectin

Con-A (either *C. ensiformis*, *Canavalia brasiliensis*, or *Canavalia virosa*) in both rhFSH starting material and fraction 4. Although a minor component of Con-A was detected in rhFSH, the band at 26–30 kDa in fraction 4 was predominantly ascribed to Con-A. Turning to the literature, we found that Con-A has a molecular weight of 26.5 kDa and an isoelectric point between 4.5 and 5.5, the protein size and isoelectric point values expected for FSH isolated in fraction 4. During our fractionation process, we used Con-A conjugated to sepharose. Furthermore, personal communication with the suppliers of rhFSH confirmed the use of Con-A in the purification step. A Western blot analysis using a specific polyclonal Con-A antibody also confirmed that the band migrating at 26–28 kDa in fraction 4 was Con-A (Figure 3C). Therefore, to elucidate the origin of the Con-A in the FSH fraction causing cell death, we tested the effect of commercial nonconjugated and sepharose-conjugated Con-A in primary cultures of

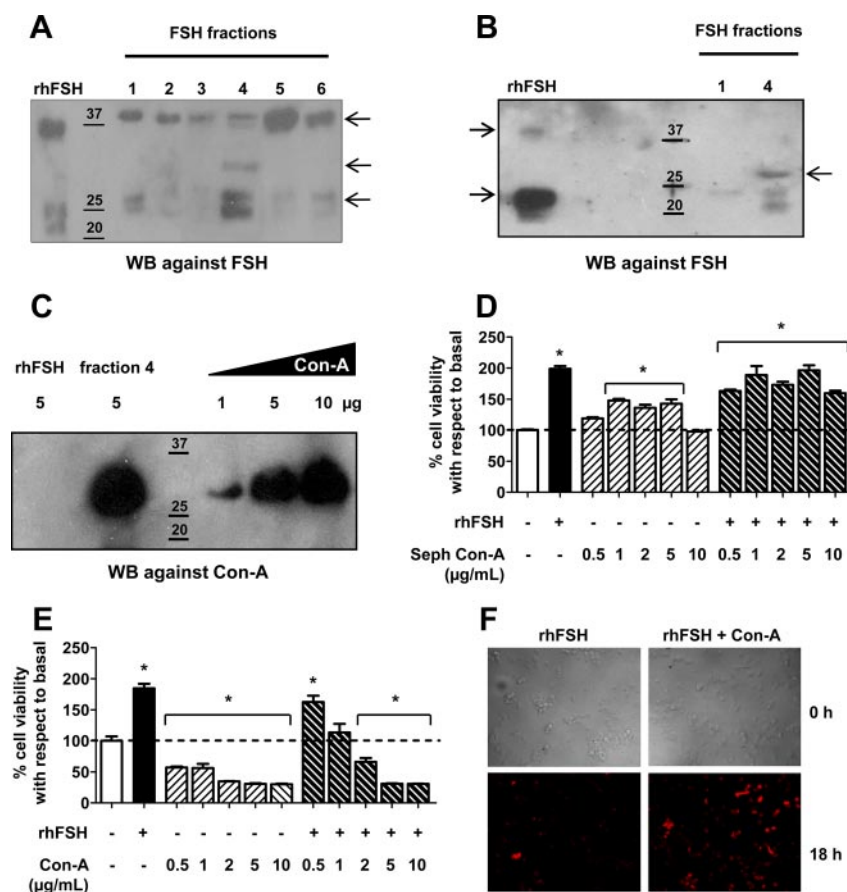


Figure 3. Con-A is present in FSH-fraction 4 and promotes rat granulosa cell death. All 6 FSH fractions were analyzed by Western blotting (WB) using a polyclonal FSH antibody (A). Two common bands of native (35–37 kDa) and denatured forms (22–25 kDa) of FSH were observed in all FSH fractions. In fraction 4, an exclusive band migrating at 26–28 kDa was observed. This band in fraction 4 did not alter its migration when analyzed under reducing conditions (B), suggesting that this protein is not FSH. Using LC-MS/MS technique, the presence of FSH in each band observed in fraction 4 was confirmed. However, analysis also revealed that the unique band at approximately 26–28 kDa principally corresponded to the lectin, Con-A. Western blotting using a polyclonal Con-A antibody confirmed that the band migrating at 26–28 kDa in fraction 4 was Con-A (C). To determine whether the cell death-inducing capacity of fraction 4 was due to the presence of Con-A, the effect of 2 commercial forms of this lectin was assessed. Primary cultured rat granulosa cells were incubated for 48 hours with sepharose-conjugated Con-A (seph Con-A), which was used for affinity chromatography during our FSH fractionation (D) or nonconjugated Con-A (E). The MTS assay demonstrated that cell death was observed only in the presence of nonconjugated Con-A, and this was further confirmed by TUNEL analysis (F, upper panels: contrast phase; lower panels: fluorescence, TUNEL). Data are presented as mean \pm SE (n = 3). * ANOVA $P < .05$ in comparison with basal condition.

rat granulosa cells. As shown, sepharose-conjugated Con-A does not affect cell viability in a range of concentrations (Figure 3D). However, nonconjugated Con-A brings about a significant decrease in cell viability (Figure 3E) and an increase of TUNEL-positive cells (Figure 3F). Moreover, nonconjugated Con-A appears to compete with FSH, preventing its trophic effect. Therefore, we conclude that in our fractionation process, we have concentrated the minute levels of Con-A left over from the recombinant FSH preparation. However, now turning the emphasis to Con-A, it was imperative to ascertain whether previous results obtained with fraction 4 can be replicated

by Con-A alone. To this end, early antral follicles were individually cultured in the presence of rhFSH, fraction 4, Con-A, or a combination of Con-A plus rhFSH (Figure 4). As previously observed with fraction 4, after 3 days of treatment, follicles incubated with Con-A demonstrated darkening, oocyte degeneration, and death of follicular cells (Figure 4A). Coincubation with a combination of Con-A plus rhFSH was associated with an intermediate response, with follicles showing initial signs of atresia (as pyknosis of granulosa cells and loss of basal membrane integrity) and in some cases accompanied by initial oocyte vacuolization (Figure 4B). Conversely, healthy follicles with high homogeneity in the granulosa layer and an intact oocyte were observed in the presence of rhFSH (Figure 4C), thus confirming the trophic effects expected for the gonadotrophin. Demonstrating a pattern extremely similar to that observed with fraction 4, of the 33.3% of Con-A-treated follicles that underwent spontaneous rupture, 100% had an atretic histology (Figure 4D), whereas of the 66.7% of rhFSH-treated follicles that underwent rupture, 88.8% of these demonstrated healthy histological characteristics (Figure 4F and Table 1). Interestingly, in follicles treated with a combination of rhFSH and Con-A, an equal proportion of healthy and atretic histology (Figure 4E) was observed when spontaneous ruptured occurred.

Despite demonstrating granulosa cell death and follicular degeneration *ex vivo*, we wished to establish the *in vivo* effect for fraction 4 and Con-A in rat follicular development. To this end, rats received intrabursal administration of rhFSH in one ovary, whereas the contralateral ovary received a combination of rhFSH and fraction 4 or Con-A (Figure 5). Ovaries treated with rhFSH showed characteristic maturing follicles at varying stages of development, including atresia (Figure 5, A–C). Combinations of rhFSH and fraction 4 in contralateral ovaries demonstrated similar stage variation in maturing follicles. However, a significant increase in follicular atresia was

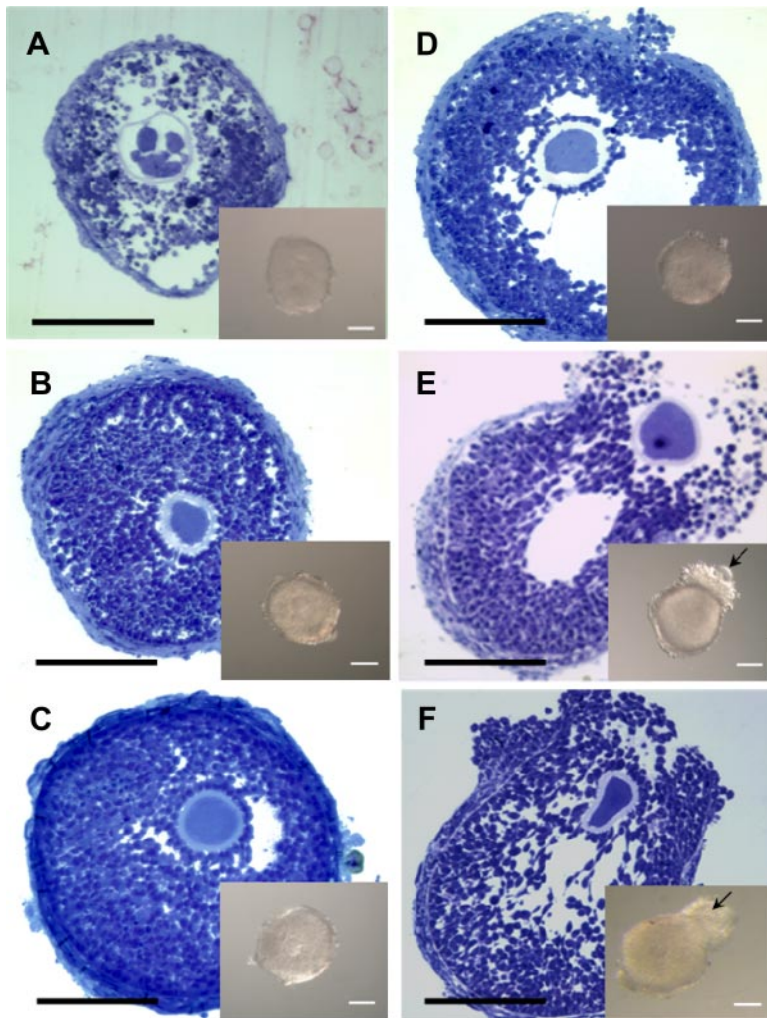


Figure 4. Nonconjugated Con-A prevents follicular maturation. Ex vivo-cultured rat early antral follicles were exposed to either rhFSH or nonconjugated Con-A or both. As observed with fraction 4, follicles incubated with Con-A demonstrated significant damage in the granulosa cell layer and oocyte degeneration (A), whereas follicles treated with a combination of lectin plus rhFSH showed initial characteristic of atresia as pyknosis and detachment of the granulosa layer from the basal membrane (B). A healthy oocyte and high homogeneity in the granulosa layer were present in follicles treated with rhFSH (C). When spontaneous rupture occurred, follicles incubated in the presence of Con-A showed scarce granulosa cells release, and subsequent histological analysis revealed oocyte vacuolization accompanied by granulosa cell degeneration and thecal hypertrophy (D). In a combination of rhFSH + Con-A, most follicular cells and the oocyte looked healthy. However, the cumulus that normally surrounds the oocyte was not observed, suggesting an abnormal process (E). Conversely, in follicles incubated with rhFSH, a brilliant expanded cumulus was present along with a histology similar to that of normal ovulation when rupture occurred (F). Representative images from each treatment were selected. In each panel, follicles with similar morphology are shown in both light microscopy (large image) and stereomicroscope image (inset). Scale bar, 100 μ m; arrows indicate oocyte localization after rupture.

present, accompanied by a decrease in early antral follicles (Figure 5, A and D). Interestingly, the combination of rhFSH and Con-A promoted an identical response in terms of a significant decrease in early antral follicles and an increase in atresia (Figure 5, B and E), thus confirming that the cell death observed with fraction 4 is mediated by Con-A. It is important to recount that 9/9 animals in the rhFSH/fraction 4 group and 6/6 animals in the rhFSH/

Con-A group showed an increase in atretic follicle proportion. None of the animals treated with rhFSH showed an increase in atresia when compared with baseline. Furthermore, it is noteworthy that the follicular degeneration observed in ovaries treated with FSH fraction 4 or Con-A is indistinguishable from the atresia observed in rhFSH-treated ovaries.

As a preliminary approach to how Con-A mediates its effect, we evaluated ovarian proliferation and angiogenesis (Figure 6). Measuring proliferation by the staining intensity of PCNA, we observed a significant decrease in rhFSH-induced proliferation in the presence of Con-A (Figure 6A, left panel; compare panels C and E). Furthermore, the same result was observed with fraction 4 in combination with rhFSH (Figure 6B, left panel; compare panels C and G). Exploiting the presence of Von Willebrand factor as an endothelium marker, we analyzed differences in staining for this protein in the treated rat ovaries. In both, treatment with Con-A (Figure 6A, right panel; compare panels D and F) or fraction 4 (Figure 6B, right panel; compare panels D and H) reduced protein staining in blood vessels and ovarian relative vascular area were observed.

Given that we have observed the potential of Con-A to bring about cell death in granulosa cells, ex vivo-cultured follicles, and to increase the incidence of atresia in vivo, we asked the question whether the rat ovary possessed binding sites for this lectin. To this end, we analyzed lectin stain-

ing in rat ovary cross-sections that had undergone DES priming. As shown, there exists weak staining in the presence of labeled Con-A (Figure 7, A–C, corresponding to increasing magnifications). Interestingly, Con-A staining increased in granulosa cells and notoriously in the vascular network when the ovary had been stimulated with rhFSH (Figure 7, E–G, corresponding to increasing magnifications). As control of the technique, nonspecific staining

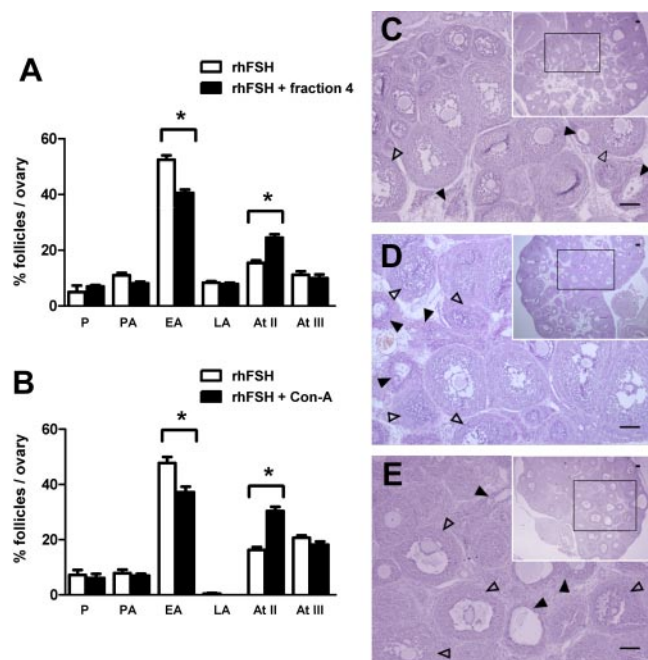


Figure 5. FSH-fraction 4 and Con-A decrease the numbers of healthy early antral follicles and increase follicular atresia. DES-primed rats were treated by intrabursal administration with rhFSH or a combination of rhFSH plus fraction 4 ($n = 9$) or nonconjugated Con-A ($n = 6$) in the contralateral ovary. At 48 hours, the ovaries treated with rhFSH + fraction 4 demonstrated a significant increase in follicular atresia accompanied by a notable decrease of healthy follicles at the early antral stage in comparison with rhFSH-treated ovaries (A). A similar result was observed in the presence of rhFSH + Con-A (B), strengthening the theory that fraction 4-mediated cell death is derived from Con-A. Representative images from control rhFSH (C-), rhFSH + fraction 4 (D-), and rhFSH + Con-A (E)-treated ovaries are shown. Insets show images at lower magnification. Follicular stage categories: P, primordial + primary (unilaminar); PA, preantral; EA, early antral; LA, large antral; At II, initial-to-mid atretic stage; At III, advanced atretic stage. Atretic follicles stage II (white arrowhead) and III (black arrowhead) are indicated in the images. Scale bar, 100 μ m. Data are presented as mean \pm SE. *, paired Student's t test $P < .01$.

was assessed by slide preincubation with methyl- α -D-mannopyranoside before lectin addition (Figure 7, D and H).

Discussion

Here, we describe a fraction of purified recombinant FSH that is capable of bringing about cell death of granulosa cells and preventing the maturation of antral follicles in a rat model. Further analysis of this fraction revealed that during the process of fractionation by IEF and affinity chromatography, we had concentrated the lectin Con-A into this fraction. Literature analysis of the isoelectric point of Con-A demonstrated that this lectin would concentrate into fraction 4, and the presence in the nonretained elution peak of our lectin affinity column is in accordance with this lectin not binding to itself. By mass

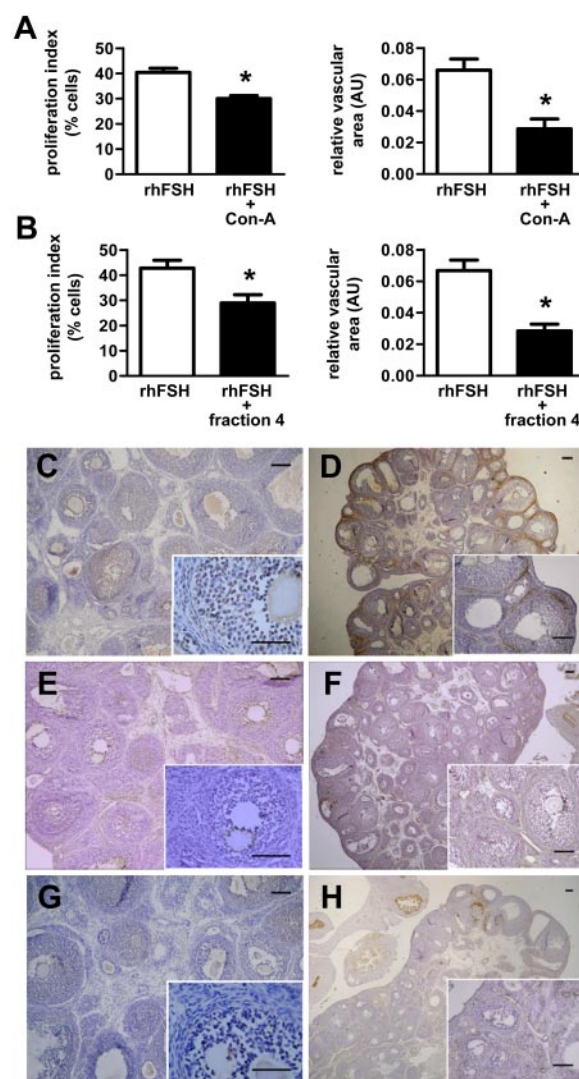


Figure 6. FSH-fraction 4 or Con-A decreases granulosa cell proliferation and angiogenesis in the rat ovary. PCNA and Von Willebrand factor protein were used as markers of the effect of fraction 4 and Con-A on proliferative activity and angiogenesis. The proliferation index, which was measured by PCNA presence in the granulosa cell compartment from antral follicles, was diminished in the presence of nonconjugated Con-A (A, left). Similarly, the relative vascular area index (as measured by Von Willebrand factor expression) was significantly reduced in the presence of nonconjugated Con-A (A, right). The combination of rhFSH plus fraction 4 also demonstrated a significant reduction in proliferation index (B, left) and relative vascular area (B, right). Representative images from rhFSH (C and D-), rhFSH + Con-A (E and F-), and rhFSH + fraction 4 (G and H)-treated ovaries are shown. (C, E, and G) PCNA. (D, F, and H) Von Willebrand factor. Insets show images containing healthy antral follicles at higher magnification. Proliferation index: PCNA-positive cells expressed as a percentage of the total number of cells. Vascular area: Von Willebrand factor-positive cells expressed as area occupied by blood vessels respect to the total area of the ovary. AU, arbitrary units. Scale bar, 100 μ m. Data are presented as mean \pm SE ($n = 5$). *, paired Student's t test $P < .01$.

spectrometry, we identified this lectin in the starting FSH preparation, and we are further confident that this contaminant was carried over from the recombinant FSH preparation, because our sepharose-conjugated Con-A

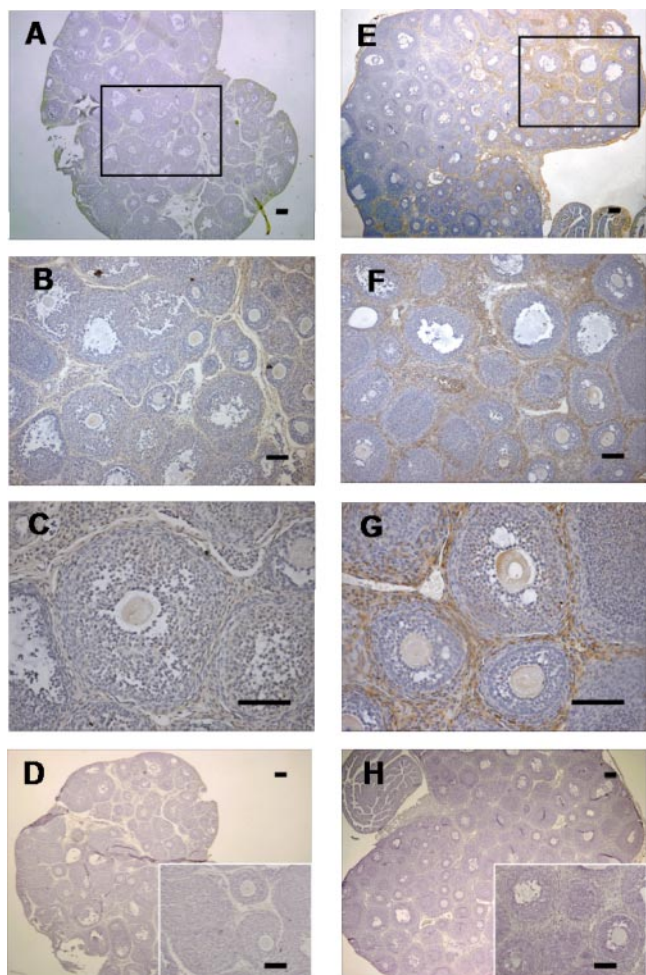


Figure 7. Locally administrated rhFSH induces an increase of Con-A high-affinity sites in the rat ovary. DES-primed rats were treated by intrabursal administration with rhFSH or saline solution. By immunohistochemical analysis, the presence of Con-A affinity sites was detected (albeit weakly) in granulosa, theca, and ovarian stroma under basal conditions (A–C). After FSH stimulation, a high-intensity stain was observed throughout the ovary, indicating an increase in Con-A affinity sites (E–G). Interestingly, intense staining was observed in the vascular network surrounding the follicles. Representative images for each condition (A and E) and higher-magnification images of selected areas are shown. Negative controls for untreated (D) and rhFSH-treated ovaries (H) were obtained by preincubation of slides with methyl- α -D-mannopyranoside before lectin incubation and immunohistochemical analysis; insets show images at higher magnification. Scale bar, 100 μ m.

did not induce cell death. This small contaminant does not affect FSH biological activity, because we reported here a promotion of cell survival, proliferation, and follicular maturation. This is further demonstrated in experiments where rhFSH is compared with fraction 1, which does not have an appropriate isoelectric point to concentrate lectin, nor did it stain for lectin presence by Western blotting. Between these hormone preparations, no differences in proliferation or survival of granulosa cells was detected, nor was there an alteration in follicular maturation. However, we demonstrate in this article that insufficient re-

moval of lectin in any hormone preparation may create competition with FSH and result in a dampening or elimination of clinical response. Furthermore, our observations do not discard that variations in FSH glycosylation may contribute to varying biological activity of commercial FSH preparations. Although we cannot speculate that lectin contamination is the cause, there is accepted clinical observation that batches of rhFSH exist that are not as effective to stimulate follicular development as others (41, 42).

The most important finding of this work is the observation that Con-A can induce granulosa cell death and increase follicular atresia. Interestingly, it has been recently reported that Con-A can bring about cell death in hepatic cells and cancer cells (25, 26). The literature also states that glycosylation patterns change during the menstrual cycle (43), and thus Con-A binding to molecules containing carbohydrates residues may trigger cell surface proteins, such as membrane receptors, and therefore conduce a variety of cellular responses, one of which being cell death.

An interesting observation from our *in vivo* study was that Con-A (and fraction 4) lowered only the early antral follicles, while increasing the number of atretic follicles. Although it is generally acknowledged that the preantral stage is sensitive to the action of FSH, the early antral stage is regarded as the most vulnerable stage to undergo atretic degeneration (1, 2, 44). Our results from counting follicles and immunohistochemical identification of lectin affinity sites may suggest that FSH action upon maturing follicles changes the pattern of expression of carbohydrate moieties that permit Con-A to mediate a cell death response.

A mechanism of action reported here for lectin was the reduction in granulosa cell proliferation and ovarian vascularization. It is documented that follicular development is dependent on angiogenesis to progress to maturation (45), thus lectin-induced granulosa cell death is most likely due to a reduction in the availability of proangiogenic factors normally released by these cells. However, to speculate that Con-A-induced atresia occurs due to simply lack in irrigation may not be the whole story, because only the proportion of early antral follicles and not all follicles were affected. These results suggest that carbohydrate binding within the follicle or ovary can modulate FSH-promoted follicular maturation and ovarian angiogenesis. This argument is given weight by the *ex vivo* culture of whole follicle, where in the presence of highly enriched medium, it does not prevent the ability of Con-A to induce follicular cell death. Furthermore, the increase in lectin staining after FSH stimulation suggests that this hormone is modifying glycosylation in the rat ovary, and this is associated with follicular fate. Although an increase in lectin binding sites was observed in the granulosa cells, the notorious change

was associated to the ovarian vascular network. The consequences of this are currently unresolved, and thus future experiments will examine the changes in glycosylation and its biological consequence in the ovary and throughout the estrous cycle.

It is well established that the communication between follicular cells and the oocyte is essential for development and maturation of competent oocytes (46, 47). A result that caught our attention was that *in vitro* lectin-treated follicles occasionally released an intact or fragmented denuded oocyte. Although no mechanism has been associated, this phenomenon has been observed before and associated to infertility (48, 49) or oocyte abnormalities as arrest in prophase, and thus cannot be regarded as an egg capable of successful fertilization (50, 51).

In summary, we have identified from a recombinant FSH preparation that Con-A can reduce follicular viability. Numerous clinical applications are suggested by these findings. It remains to be determined what are the physiological consequences of circulating lectin-like molecules in fertility and infertility. Furthermore, this work suggests a potential use of lectin as a nonsteroidal contraceptive and in the treatment of ovarian cancer, particularly in 4% of the ovarian cancers that are derived from granulosa cells. Finally, the demonstration that lectin increases granulosa cell death may shed light on a key role of carbohydrates in the still obscure physiological process of follicular selection and atresia.

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