High levels of mineralocorticoids in preovulatory follicular fluid could contribute to oocyte development

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Objective: To identify aldosterone and precursors in ovarian follicles and to relate levels of mineralocorticoids to previously described renin-angiotensin system follicular fluid content.

Design: Experimental.

Setting: Academic laboratory and affiliated large private practice.

Patient(s): Women undergoing oocyte retrieval for in vitro fertilization (IVF).

Main Outcome Measure(s): The concentrations of mineralocorticoids were measured in plasma and follicular fluid. Granulosa cell mRNA expression and oocyte receptor content were evaluated.

Result(s): High concentrations of preovulatory aldosterone and corticosterone were measured in follicular fluid (419.5 ± 122.2 and 218,383 ± 124,143 pg/mL, respectively). Increased mineralocorticoid levels are found in follicular fluid compared with in plasma and in large follicles compared with in small. Plasma aldosterone levels increase before ovulation. Granulosa cell gene expression that promotes aldosterone production and accumulation of corticosterone is increased in younger patients. Aldosterone receptors are localized to the surface of human oocytes.

Conclusion(s): High levels of aldosterone and its precursor, corticosterone, were found in ovarian follicles. This combined with the presence of aldosterone receptors on oocytes suggests a possible role for aldosterone in oocyte development. (Fertil Steril 2011;95:182–7. ©2011 by American Society for Reproductive Medicine.)

Key Words: Follicular fluid, aldosterone, corticosterone, mineralocorticoids, ovary, ovarian, human, renin-angiotensin system

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MATERIALS AND METHODS

Institutional Review Board Approval/Site

This study was approved by the Beth Israel Deaconess Medical Center Institutional Review Board (IRB). All patients were treated at Boston IVF, a large infertility clinic affiliated with Beth Israel Deaconess Medical Center. Informed consent was obtained from all subjects at the time of oocyte retrieval during an IVF cycle that provided granulosa cells (GCs), follicular fluid aspirates, and plasma. The IRB waived consent for the study of deidentified discarded oocytes.

Subjects

Patients undergoing oocyte retrieval for the purpose of IVF were eligible to participate (n = 44). Patients were categorized as reproductively young if...
under 35 years of age and reproductively old at age 40 years or more. All patients underwent controlled ovarian hyperstimulation as described elsewhere (18). Follicle aspirates from two to three large follicles (>16 mm) and two to three small follicles (<10 mm) were collected, processed, and stored separate from one another. All other follicle aspirates were pooled within study subjects. Plasma samples from discarded blood collection before administration of hCG (pre-hCG) and on the day of oocyte retrieval (post-hCG) were obtained when possible.

**Follicular Fluid, Plasma, and GC Processing**

Follicular fluid and plasma samples were processed as described elsewhere (18). GCs were isolated from the cellular pellet remaining after follicular fluid centrifugation. This pellet was resuspended in 0.1% trypsin solution, incubated for 10–15 minutes, and centrifuged for 20 minutes on a 45% Pure-Ception gradient. The GC rich layer was suspended in phosphate-buffered saline and archived at −80°C after the adding Qiagen RLT buffer with beta-mercaptoethanol. The mRNA purification was completed as per the Qiagen RNeasy Mini protocol (Valencia, CA).

**Aldosterone and Corticosterone Quantification**

Commercially available enzyme linked immunoassays (EIA) were used to quantitate aldosterone (Alpco Diagnostics, Salem, NH) and corticosterone (Cayman Chemical Company, Ann Arbor, MI) concentrations in follicular fluid and plasma. Controls and standards were analyzed in accordance with manufacturer recommendations and met the criteria for quality assurance. All samples were analyzed in duplicate. For the corticosterone EIA, samples were diluted (samples 1:2500, high performance liquid chromatography [HPLC] fractions 1:10) to achieve concentrations in the center of the standard curve.

**Determination of Free and Bound Aldosterone Content**

Follicular fluid samples from a subset of patients with a sufficient availability of pooled fluid were fractionated by HPLC (Pharmacia, Sweden) on a Superose 6 size exclusion column. Particle molecular weight of the molecules and their complexes was determined by light scattering using the supplier’s software (Wyatt Technology, Santa Barbara, CA). Estimation of free and bound aldosterone content was based on the estimated molecular mass of particles within specific fractions combined with the content of aldosterone as

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determined by EIA (see above). Unbound aldosterone eluted in the last fractions, corresponding to a molecular mass 360 Da. Patients who ultimately became pregnant were compared with those who did not achieve a pregnancy.

**Glanulosa Cells mRNA Microchip Analysis**

Total mRNA from pooled GCs of three young and three old patients were compared on two identical microarrays (Agilent Human Genome CGH Microarray 44K). The microarray methods were described elsewhere (19). GeneSpring software (Agilent) was used to generate lists of selected genes that differed at least twofold between the study groups. Ingenuity Systems Pathway Analysis Software (Redwood City, CA) was used to interpret the microchip data. Specifically, the software identified gene pathways that were significantly different between our two groups, which included pathways that affect mineralocorticoid content.

**Oocyte Immunohistochemistry**

Nonfertilized oocytes with intact zona pellucida were fixed with 4% paraformaldehyde and washed with 0.1% Tween/0.05% Saponin solution. Surrounding GCs were removed during these washing steps. The oocytes were blocked with a 5% horse serum/0.1% Tween/0.05% Saponin solution. Primary antibodies were applied overnight at 4°C: Abcam (Cambridge, MA); angiotensin II receptor 1 (AIIIR1, mouse monoclonal clone H3122, 1:20 dilution); and ABR aldosterone receptor (AldoR, mouse monoclonal clone H10c4c9f, 1:100 dilution). Control oocytes were incubated in filtered washing solution. After another series of washes, all oocytes were incubated at room temperature with fluorescently labeled secondary antibody (Alexa 430 nm, 1:200 dilution) and Hoechst stain (1:5000 dilution) for 2 hours. Fluorescence was then assessed by fluorescence microscopy (Zeis LM, Germany).

**Statistical Analysis**

SPSS version 15.0 (Chicago, IL) software was used for statistical analysis. The following parametric tests were used after confirming a normal distribution of data: the paired t-test for comparison of aldosterone or corticosterone content among patients who had multiple samples measured and the Pearson correlation coefficients for comparison of associations of aldosterone content with other variables.

**FIGURE 2**

Corticosterone (CS) content in follicular fluid (FF) and plasma samples is measured by EIA and presented in standard box plot format. All samples are presented in Figure 2A, and paired comparisons are presented in Figures 2B–2D. Paired t-tests were used for all comparisons. Significant differences are labeled with asterisks: **P < .001.
RESULTS

High Concentration of Aldosterone in Preovulatory Follicles

Forty-four patients had follicular fluid from large follicles analyzed for aldosterone. Among these 44 patients, additional samples were obtained and analyzed for aldosterone content: small follicles (24 patients), pre-hCG plasma (14 patients), and post-hCG plasma (21 patients). Paired comparisons (paired t-test for all paired comparisons) of samples revealed significantly higher mean concentrations of aldosterone in large follicles compared with both pre-hCG (339.5 ± 115.6 vs. 85.5 ± 33.9 pg/mL, \(P < .001\), \(n = 14\) pairs) and post-hCG plasma (343.5 ± 107.8 vs. 159.4 ± 96.7 pg/mL, \(P < .001, \text{Figure 1B}, n = 21\) pairs). A small, but statistically significant elevation of aldosterone content was seen in large follicles compared with small follicles \(485.4 ± 86.4\) vs. \(397.7 ± 137.4\) pg/mL, \(P = .001, \text{Figure 1C}, n = 24\) pairs). Finally, plasma aldosterone concentration was found to increase after administration of hCG (85.5 ± 33.9 vs. 179.2 ± 92.5 pg/mL, \(P = .001, \text{Figure 1D}, n = 14\) pairs).

The aforementioned comparisons are shown in standard box plot format in Figure 1 to illustrate the distribution of data and to highlight clear differences in aldosterone content between samples. Of note, the aldosterone level in follicular fluid was markedly higher than the level in the matched plasma samples. The aldosterone content in the follicular fluid was also found to be markedly higher than the plasma reference range (mean, 105 pg/mL; range, 25–315 pg/mL). The content in the plasma samples was found to be generally within the range.

Aldosterone level weakly correlated with some demographic and outcome variables. Higher levels of aldosterone were associated (Pearson’s correlation coefficient for all associations) with younger age \((R = -0.185)\), greater body mass index \((R = 0.286)\), higher number of oocytes retrieved \((R = +0.179)\), and higher peak E2 levels \((R = +0.111)\). Plasma and follicular fluid aldosterone levels were strongly correlated \((R = +0.881)\).

High Concentration of Corticosterone in Preovulatory Follicles

Corticosterone content in the follicular fluid paralleled the aldosterone findings above. Forty patients had follicular fluid samples from large follicles analyzed for presence of the aldosterone precursor corticosterone. Among these 40 patients, additional samples were assayed for corticosterone content in small follicles \(25\) patients), pre-hCG plasma \(16\) patients), and post-hCG plasma \(12\) patients). The mean concentration of corticosterone was significantly increased in the large follicles compared with matched pre-hCG plasma \(245,693 ± 161,774\) vs. \(12,170 ± 8,790\) pg/mL, \(P < .001, \text{n = 14}\) pairs) and post-hCG plasma \(245,693 ± 161,774\) vs. \(9,404 ± 5,921\) pg/mL, \(P < .001, \text{Figure 2B}, n = 12\) pairs). The large follicles had significantly more corticosterone than the small follicles \(220,364 ± 92,812\) vs. \(161,774 ± 139,571\) ± 114,318 pg/mL, \(P < .001, \text{Figure 2C}, n = 25\) pairs). There was no difference in corticosterone content between pre-hCG and post-hCG plasma samples \(12,170 ± 8,790\) vs. \(9,404 ± 5,921\) pg/mL, \(P = .318, \text{Figure 2D}, n = 12\) pairs). As with aldosterone, corticosterone concentration in the follicular fluid was higher than the reference range \((900–3,900\) pg/mL). The median corticosterone concentration in follicular fluid was approximately 50 times the upper limit of the reference range, and median plasma concentration was approximately double the upper limit.

Free and Bound Aldosterone Increased in Pregnant Patients

The free and bound content of aldosterone among three patients who achieved a pregnancy was increased compared with between two
patients who did not achieve a pregnancy for the majority of eluted fractions. Specifically, the profile for the pregnant group had a higher proportion of aldosterone bound to large complexes (fractions with estimated molecular mass of 213–451 kDa) and higher free aldosterone (fraction with estimated molecular mass of 360 Da) compared with nonpregnant patients. The mean age, number of follicles, and cycle number were similar for the two groups, but peak E2 was higher in the pregnant group (pregnant: 35 years, 7.7 follicles, 2.3 cycles, 2,266.7 pg/mL; not pregnant: 33 years, 7.5 follicles, 2.0 cycles, 1,051 pg/mL).

**Granulosa Cells mRNA Analysis**

The total RNA from GCs of three young and three older patients was compared by a differential gene expression profiling by microgene chip analysis. Comparing the level of mRNA content in young versus old patients favored production of aldosterone and the accumulation of corticosterone in young patients. The magnitude of changes and the specific gene functions (20–34) for differentially expressed mRNAs are summarized in Table 1. Of note, the mRNA expression in the corticosterone quantity pathway is primarily associated with genes that inhibit accumulation. The higher mRNA content of these genes in old patients is therefore associated with decreased corticosterone accumulation. These data support the hypothesis that specific coordination in the level of expression of genes that enhance aldosterone production and corticosterone accumulation occurs in the young patients who have the best fertility prognosis.

**Oocyte Immunohistochemistry: Aldosterone Receptor Presence on Human Oocytes**

Immunohistochemistry for the aldosterone receptor (AldoR) revealed a strong signal in comparison with controls (Figure 3). Specific weaker staining was also seen with the AIIR1 antibody. Both AldoR and AIIR1 staining were seen diffusely over the oocyte. Based on concomitant Hoechst staining, the receptors did not appear to be localized to the nucleus but more likely are on the oocyte membrane or within the cytoplasm. The exact site of the receptors was unclear.

**DISCUSSION**

This is the first report of the presence of mineralocorticoids in the human ovarian follicle. Specifically, high levels of aldosterone and its precursor corticosterone are found in the follicular fluid of women undergoing IVF. Content is significantly increased in follicular fluid compared with in plasma, and peak levels are noted before ovulation. Additionally, we report preliminary evidence of local aldosterone synthesis and aldosterone receptors on the oocyte. Our observations are novel findings in ovarian biology and add to prior knowledge of an OvRAS.

Previously published studies revealed the presence of an OvRAS that is most active during the preovulatory time period (1–8), mimicking the elevations in mineralocorticoids described here. Outside of the ovary, aldosterone is an active component of the renin-angiotensin system system and is tightly regulated by angiotensin II (35). The same regulation scheme may be active in the ovary. Further, OvRAS with aldosterone may play a role in folliculogenesis or oocyte maturation. The increased aldosterone in preovulatory follicular fluid, in larger follicles most likely to contain mature oocytes, and among younger patients with more oocytes are some observations that are consistent with this claim. Alternatively, aldosterone level may be influenced by another undetermined factor. For example, estrogen is weakly correlated with aldosterone in our study.

The site of aldosterone synthesis is unclear. In our study, we demonstrated both higher levels of aldosterone in follicular fluid compared with in plasma and a differential expression of genes associated with aldosterone production and corticosterone accumulation in GCs of younger patients. Although these observations are consistent with ovarian production of aldosterone, there are some inconsistencies. We did not demonstrate a difference in CYP11B2 (aldosterone synthase) mRNA expression, a critical enzyme in the synthesis pathway for aldosterone. Future studies designed to specifically address the question of the site of aldosterone synthesis will provide much needed information. Data supporting ovarian synthesis would challenge the classic dogma that mineralocorticoids are produced exclusively in the adrenal gland (35) and provide some explanation for clinical examples of rare aldosterone-secreting tumors in the ovary (36–38).

In summary, this is the first study to demonstrate high levels of mineralocorticoids in the preovulatory ovarian follicular fluid.
of humans. Aldosterone may be the active component of the OvRAS, conferring an improved fertility prognosis with the attainment of sufficient follicular levels. Further study on the site of aldosterone production and its role within the ovary is needed.

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REFERENCES