PHYSIOLOGY

The Graafian Follicle Is a Site of L-Ascorbate Accumulation

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Purpose: *Our purpose was to evaluate the* L-*ascorbate level in human preovulatory follicular fluid and to quantify the blood/follicle gradient for vitamin C. The effect of smoking on the follicular* L-*ascorbate concentration was studied. The correlations were tested between follicular* L-*ascorbate and follicle size and oocyte maturity.*
Methods: *In 65 women undergoing in vitro fertilization, samples of follicular fluid and blood serum were collected. Biochemical analyses included* L-*ascorbate determinations by a colorimetric method and cotinine measurements by a radioimmunoassay.*
Results: *The average follicular fluid:serum ratio for* L-*ascorbate was 1:68. Ascorbate levels in follicular fluid and serum were significantly correlated. The follicular* L-*ascorbate level did not correlate with the follicle size and the oocyte maturity grade. Insignificantly lowered follicular* L-*ascorbate levels were observed in smokers.*
Conclusions: *The extracellular compartment of the Graafian follicle is a site of an ascorbate accumulation. Exposure to tobacco smoke does not significantly diminish the intrafollicular pool of* L-*ascorbate.*

KEY WORDS: L-ascorbate; follicular fluid; fertilization *in vitro*; smoking; cotinine.

INTRODUCTION

Due to an ancient gene mutation, humans are among very few species unable to synthesize L-gulonolactone oxidase, a key enzyme for L-ascorbic acid biosynthesis (1). Humans are, therefore, dependent on the dietary supply of this vitamin. It has been believed for a long time that vitamin C is essential for the reproductive integrity of primates (2). However, the exact role of L-ascorbate inside the ovarian follicle remains obscure.

Follicular fluid (FF) creates a highly specific milieu required for the maturation of both the granulosa cells and the oocyte (3). The vitamin composition of this microenvironment has not been fully described to date. The available literature contains only one preliminary report on L-ascorbate measurements in the human follicular fluid (4).

The aim of the present study was to evaluate the L-ascorbate levels in human preovulatory FF and to quantify the blood/follicle gradient for this vitamin. The relationship between the level of tobacco smoke exposure and the determined follicular L-ascorbate levels was studied. Moreover, the correlations between FF L-ascorbate and follicle size and oocyte maturity were tested.

MATERIALS AND METHODS

Subjects and Treatment

The study group consisted of 65 women treated with in vitro fertilization (IVF)–embryo transfer (ET) at Brigham and Women's Hospital, Boston, MA, USA. The age of the patients averaged 36 years (range, 28–42 years). Etiologic factors for infertility included tubal obstruction (26 patients), male factor (15 cases), endometriosis (6 patients), unexplained infertility (6 cases), polycystic ovarian syndrome (6 patients), uterine factor (3 patients), and cervical factor (3 cases).

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Controlled ovarian hyperstimulation was achieved using a combined treatment with gonadotropin-releasing hormone analogue (Lupron; TAP Pharmaceuticals, Deerfield, IL) and human menopausal gonadotropin (Pergonal; Serono Laboratories, Randolph, MA) according to previously described protocols (5). Transvaginal ultrasound and estradiol (E_2) measurements were used to monitor the IVF cycle, and 10,000 IU of human chorionic gonadotropin (hCG) (Profasi; Serono Laboratories) was administered intramuscularly when two or more follicles were evident whose diameter equaled or exceeded 18 × 12 mm and the serum E_2 concentration was 600 pmol/ml or greater (conversion factor to SI units, 3671). Oocytes were obtained approximately 36 hr after the hCG stimulus with the use of transvaginal ultrasound-directed ovum retrieval (TUDOR).

Specimen Collection

Sixty-five samples of FF originating from 65 individual follicles were collected from 65 patients during TUDOR. The follicular aspirates were examined microscopically, and when the oocyte–corona–cumulus (OCC) complexes had been identified, they were removed from the FF and processed for insemination. The maturity grade of the OCC complexes was assessed according to the criteria described by Veek (6). Only first-puncture clear FF samples free from contamination with blood or medium were included in the study. FF samples were centrifuged at 1000*g*, and the cell-free liquid was used for laboratory analysis.

An aliquot of venous blood was obtained from all the patients approximately 10 min before TUDOR. Clotted blood was then centrifuged twice (1500*g*, 10 min) to yield clear serum.

Fresh samples of serum and FF were analyzed for L-ascorbate content, within 2 hr of collection. The remaining aliquots of biological material obtained were stored at −20°C until assayed for cotinine content.

Measurement of L-Ascorbate in Serum and FF

We used a kit to measure L-ascorbate (Boehringer Mannheim Corp.) in which L-ascorbate reduces the tetrazolium salt MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] in the presence of the electron carrier PMS (5-methylphenazinum methyl sulfate) at pH 3.5 to MTT-formazan. In a cuvette with the sample the sum of the reducing substances is measured. For the specific determination of L-ascorbate, in a sample blank determination only the L-ascorbate fraction as a part of all reducing substances present in the sample is oxidatively removed by L-ascorbate oxidase (AAO) in the presence of oxygen. The dehydroascorbate formed does not react with MTT/PMS. The absorbance difference of the sample minus the absorbance difference of the sample blank is equivalent to the quantity of L-ascorbate in the sample. The MTT-formazan is the measuring parameter and is determined by means of its absorbance in the visible range at 578 nm.

Measurement of Cotinine in FF

The detection of smoking through the measurement of nicotine metabolites such as cotinine is favored over other methods, such as carboxyhemoglobin or thiocyanate determinations (7). This is because cotinine is tobacco specific. Moreover, cotinine has a half-life of approximately 11 hr, while nicotine and carboxyhemoglobin have much shorter half-lives.

Cotinine concentrations in FF were measured by radioimmunoassay (Double Antibody Nicotine Metabolite RIA Kit; Diagnostic Products Corporation, Los Angeles, CA). The double antibody procedure is a liquid-phase radioimmunoassay, wherein ^{125}I-labeled cotinine competes for a fixed time with the cotinine in the patient's sample for antibody sites. After incubation for a fixed time, the separation of bound from free is achieved by the PEG-accelerated double antibody method. Finally, the antibody-bound fraction is precipitated and counted. Patient sample concentrations are read from a calibrations curve.

As cotinine easily crosses the blood/follicle barrier, its determinations in FF reflect the individual's exposure to tobacco smoke in a dose-dependent manner (8). We used recently published criteria (9) to divide the subjects into three exposure groups according to the cotinine level in FF (C-FF): nonsmokers [C-FF, ≤20 ng/ml (conversion factor to SI units, 5.68)], passive smokers (C-FF, 20–50 ng/ml), and active smokers (C-FF, >50 ng/ml).

For the laboratory determinations described above, all samples were analyzed in duplicate. The coefficients of variation for the tests were as follows: L-ascorbate, 8.9%; and cotinine, 9.9%.

Statistical Analyses

All data are expressed as medians and ranges of values. Statistical analysis was carried out with nonparametric methods such as the Kruskal–Wallis one-way

Table I. Concentration of L-Ascorbate (μM) in Preovulatory Follicular Fluid and Blood Serum (Descriptive Statistics)[a]

Parameter	Range	Arith. mean	SE	CV (%)	Median	Skewness
A-FF	33.40–356.00	98.40	6.98	57.2	93.94	1.97
A-S	31.30–142.80	64.61	2.86	35.7	62.05	1.43

[a] A-FF, L-ascorbate level in follicular fluid (μM); A-S, L-ascorbate level in blood serum (μM); CV, coefficient of variation.

analysis of variance by ranks, Wilcoxon signed rank test, and Spearman's rank correlation test. StatView 512+ software for Power Macintosh (Brain-Power Inc., Agoura Hills, CA) was used for statistical processing of the data. A type I error of less than 0.05 was considered to be a significant difference.

RESULTS

The results of the L-ascorbate determinations are presented in Table I. The determined follicular L-ascorbate levels appeared to be relatively high. In all the examined samples of FF the concentration of L-ascorbate was higher than the respective level in blood serum. The median value of the FF/S ratio for L-ascorbate levels amounted to 1.68 (range: 1.26–3.01) and this intercompartmental gradient was highly statistically significant (Wilcoxon test: $Z = -7.01$, $P < 0.001$).

As shown in the scattergram (Fig. 1), the correlation between the L-ascorbate levels measured in serum and FF was statistically significant (Spearman test: $Z = 5.811$, $\rho = 0.73$, $P < 0.001$).

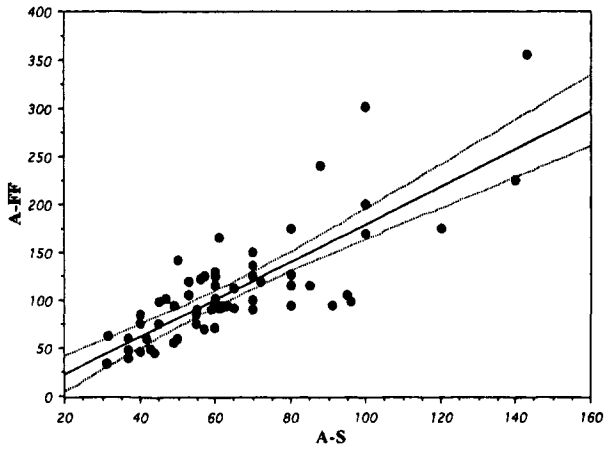

Fig. 1. Correlation between serum and follicular fluid L-ascorbate levels. Regression line and 95% confidence bands for the mean are displayed. A-FF, L-ascorbate level in follicular fluid (μM); A-S, L-ascorbate level in blood serum (μM).

The volume of the aspirated FF ranged from 2.3 to 6.8 ml (median: 4.4 ml). The ascorbate concentration in the FF was not significantly correlated with the follicle's volume.

Twenty-seven follicles of the total 65 punctured (41.5%) resulted in oocyte recovery. The presence or absence of an oocyte in the follicular aspirate had no significant relationship with the L-ascorbate content in the FF.

The follicular L-ascorbate levels were analyzed in relation to oocyte maturity. Of the 27 OCC complexes identified in the studied follicular aspirates, 15 (55.5%) were graded as metaphase I (intermediary) and 12 (44.4%) were assessed as metaphase II (preovulatory). The L-ascorbate content in the FF containing an oocyte with uncompleted I meiotic division (median, 92.27 μM; range, 51.15–190.87) did not differ significantly from that characterizing FF with an oocyte in the metaphase stage of the second meiotic division [87.01 (51.32–356.00)].

Table II compares concentrations of follicular ascorbate in patients with different levels of tobacco smoke exposure.

A tendency toward a lowered L-ascorbate level in the FF of smokers has been observed. This trend, however, did not reach the criterion of statistical significance (Kruskal–Wallis test; $P = 0.091$).

DISCUSSION

Our study shows that a mature ovarian follicle is a site of L-ascorbate accumulation. As the L-ascorbate

Table II. Follicular Fluid Ascorbate in Patients with Different Levels of Tobacco Smoke Exposure

C-FF[a] (ng/ml)	N	L-Ascorbate level in FF (μM)[b]
≤20	25	107.92 (34.01–356.00)[b]
20–50	22	96.18 (40.10–149.02)
>50	18	87.22 (33.40–148.19)

[a] Cotinine content in follicular fluid.
[b] Median (range).

is readily water-soluble, it passes freely into all compartments of extracellular water (10). A strong correlation between the serum and the FF L-ascorbate levels suggests that the size of the L-ascorbate pool in circulating blood is a major determinant of the blood/follicle transfer of this vitamin. In all the examined patients, the serum L-ascorbate levels were within normal limits. In patients with a suboptimal L-ascorbate intake, the bioavailability of this vitamin inside the Graafian follicle may be diminished.

Apart from the diet, the circulating pool of L-ascorbate may be depleted by lifestyle factors such as smoking. In smokers, serum and tissue L-ascorbate levels are diminished (11). Our results show that smoking does not lead to a significant depletion of the intrafollicular L-ascorbate reserve. Such an effect may, however, be seen in patients with a low dietary vitamin C intake.

A concentration ratio of approximately 1.7 between FF and blood serum suggests active transport of this molecule against the concentration gradient. In their preliminary report, Luck *et al.* have also found higher asorbate levels in FF than in blood serum (4). Leukocytes are considered the main cellular carriers of ascorbate (12). In the periovulatory period a massive leukocyte infiltration of the follicular wall takes place (13). Therefore, leukocytes at the blood–follicle interface are most likely the predominant source of ascorbate in the follicular milieu.

A relatively high bioavailability of L-ascorbate inside the Graafian follicle suggests an important biological role of vitamin C in this compartment. Due to the extremely high reducing potential of the ascorbate/dehydroascorbate system, vitamin C plays an important role in processes such as collagen turnover, steroidogenesis, and antioxidative defense (4). All these actions of L-ascorbate are of particular relevance to folliculogenesis and ovulation.

Ascorbate serves as a main electron donor during the hydroxylation of proline and lysine residues of procollagen (14). Since normal collagen turnover is essential for ovarian tissue remodeling (15), a high bioavailability of L-ascorbate is needed inside the preovulatory ovarian follicle. In guinea pigs with experimentally induced scurvy, degeneration of the basal lamina inside the ovarian follicles was found (16). Ascorbate is also essential for the synthesis of proteoglycans by fibroblasts of the follicular wall (17). Proteoglycans of the FF are responsible for its optimal physicochemical properties (18).

Hydroxylation steps of steroidogenesis appear to be ascorbate dependent (19). The highest concentrations of L-ascorbate were found in granulosa cells and corpus luteum (6), e.g., in the ovarian structures of excessive steroidogenic activity. Since *stratum granulosum* in the preovulatory follicle does not have direct contact with the intravascular compartment, FF serves as a natural "culture medium" for granulosa cells. The accumulation of L-ascorbate by a mature follicle may therefore be necessary for normal steroid production by preovulatory granulosa cells.

Vitamin C is a major representative of the nonenzymatic antioxidative system in the water-soluble compartment (20). Oocytes and granulosa cells are exposed within their intrafollicular microenvironment to oxygen-free radicals (21). The antioxidative potential of the FF serves as a protection against cellular injury mediated by the reactive oxygen species of both endogenous and exogenous origin (22).

Our results show that in women with a normal vitamin C intake, the follicular L-ascorbate level does not reflect oocyte maturity. Since the follicular aspirates studied originated from large codominant follicles, we were able to compare only two maturation classes of oocytes, namely, preovulatory and intermediate. Further studies including follicles yielding extremely immature or atretic ova are needed to reveal the exact role of follicular L-ascorbate in the oogenesis in humans.

Despite over 50 years of research on the reproductive functions of ascorbic acid, our knowledge of its role in the physiology of human ovary is far from complete. Further studies on the sources, metabolism, and actions of L-ascorbate inside the Graafian follicle are needed, but evidence accumulated to date strongly suggests that L-ascorbate is one of the key factors in human folliculogenesis.

CONCLUSIONS

Our results show that the extracellular compartment of the Graafian follicle is a site of L-ascorbate accumulation. The L-ascorbate concentrations in FF and blood serum were significantly correlated. The L-ascorbate levels in follicles yielding metaphase-I oocytes did not differ significantly from those containing metaphase-II ova. In women with an appropriate intake of vitamin C, exposure to tobacco smoke does not significantly diminish the intrafollicular pool of L-ascorbate.

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