Ferroportin mRNA is down-regulated in granulosa and cervical cells from infertile women

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Objective: To explore the relationship between iron and infertility by investigating iron-related gene expression in granulosa and uterine cervical cells.
Design: Case-control study.
Setting: Two tertiary hospitals.
Patient(s): Two independent cohorts of fertile (n = 18 and n = 17) and infertile (n = 31 and n = 35) women.
Intervention(s): In vitro fertilization.
Main Outcome Measure(s): Gene expression levels of ferritin light chain (*FTL*), ferritin heavy chain (*FTH*), transferrin receptor (*TFRC*), and ferroportin (*SLC40A1*) mRNA were analyzed in granulosa and cervical cells.
Result(s): In the first cohort, fertile and infertile women were similar in body mass index. Ferroportin mRNA levels were decreased in granulosa cells from infertile women in parallel with increased serum hepcidin levels. A positive association between ferroportin and *TFRC* mRNA, a gene associated with intracellular iron deficiency, was observed only in granulosa cells from fertile women. The major findings were replicated in a second independent cohort.
Conclusion(s): Ferroportin mRNAs and circulating hepcidin identify a subset of infertile women and may constitute a target for therapy. (Fertil Steril® 2017;107:236–42. ©2016 by American Society for Reproductive Medicine.)
Key Words: Ferroportin, ferritin, ovarian granulosa cells, uterine cervical cells, infertility, sterility, in vitro fertilization

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Reactive oxygen species (ROS) are produced in humans under physiologic conditions. Free radicals play a key role in the origin of life and biologic evolution. ROS are involved in physiologic processes linked to female reproduction, such as folliculogenesis, oocyte maturation, ovulation, corpus luteum formation, endometrial cycle, luteolysis, implantation, embryogenesis, and pregnancy (1). Imbalance in homeostatic control of ROS exposure causes oxidative stress, which can affect fertilization and induce apoptosis.

The importance of iron-induced ROS production and lipid peroxidation in the pathophysiology of male infertility has been documented (2). Uncomplexed iron together with superoxide, which reduces Fe^{3+} and hydrogen peroxide, provides a lethal mixture

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containing ROS that can directly damage DNA, lipids, and proteins (3). Very recently, high seminal iron was found in patients with transfusion-dependent thalassemia associated with azoospermia or oligospermia (4). Elevated systemic non–transferrin-bound iron and labile plasma iron, together with low seminal glutathione, in these patients, which correlate with sperm motility, strongly suggest that iron-induced oxidative stress may have a major role in subfertility (4).

Despite this evidence, the potential role of iron excess in female infertility has been less well studied. In women with thalassemia major, infertility seems to be attributed to iron deposition and iron-induced oxidative stress in various endocrine organs, such as the hypothalamus, pituitary, and female reproductive system (5). Given the pathophysiologic role of iron in women with β-thalassemia, endometriosis, and polycystic ovary syndrome (PCOS) (6–8), we designed the present observational study to test the hypothesis that the expression of iron-related genes might be deregulated in women with infertility.

MATERIALS AND METHODS

Cohort 1: Discovery

Follicular fluid and granulosa cells were collected from the ovulatory follicles of 49 euthyroid women, aged 22–38 years, undergoing oocyte retrieval at Hospital Universitari Dr. Josep Trueta and at Gynecologic Clinic Girexx (Girona, Spain). Thirty-one were infertile (unexplained primary infertility and no previous endocrine diseases or previous infertility treatments), and 18 were fertile women (as demonstrated by a history of at least one previous pregnancy or success of subsequent egg donation program) who voluntarily donated eggs.

All women were subjected to a standard short antagonist protocol with the use of subcutaneous gonadotropins and received 6,500 UI choriogonadotropin alpha or 0.2 mg triptorelin as an ovulation inductor when the leading follicle/s exceeded 16 mm in diameter. Granulosa cells and follicular fluids were harvested by means of ultrasound-guided transvaginal follicular aspiration 34–36 hours later. While the woman was under anesthesia, exocervical cytology was performed. After isolation of oocytes, follicular fluid containing granulosa cells and cervical cells suspended in physiologic saline solution were centrifuged at 1,000*g* for 3 minutes at room temperature. Cell samples were collected as a pellet. All samples were frozen at −80°C.

Every subject gave written informed consent after the purpose of the study was explained to her. The Institutional Review Board approved the protocol, so we certify that all applicable institutional regulations concerning the ethical use of information and samples from human volunteers were followed during this research.

Cohort 2: Replication

Follicular fluid and granulosa cells were collected from the ovulatory follicles of 52 euthyroid women, aged 19–43 years, undergoing oocyte retrieval at IVI Center for Reproductive Care (Seville, Spain). Thirty-five were infertile (unexplained primary infertility and no previous endocrine diseases, except for four women who had polycystic ovary syndrome and nine who had endometriosis), and 17 were healthy oocyte donors whose fertility was evidenced by the success of subsequent egg donation programs.

Women were subjected to a standard short antagonist protocol (n = 46) or to a long agonist protocol (n = 6 infertile patients) with the use of subcutaneous gonadotropins, and received 6,500 UI choriogonadotropin alfa or 0.2 mg triptorelin as an ovulation inductor when the leading follicle/s exceeded 16 mm in diameter. Granulosa cells and follicular fluids were obtained by means of ultrasound-guided transvaginal follicular aspiration 34–36 hours later.

After isolation of oocytes, the follicular fluids were pooled and granulosa cells collected with the use of the Dynabeads methodology as described previously (9). Briefly, granulosa cells were separated from erythrocytes by means of density-gradient centrifugation with the use of Histopaque 1077 (Sigma). The middle layer was collected and resuspended in red blood cell lysing buffer (Hybri-max; Sigma). Granulosa cells were then recovered and incubated with magnetic beads coated with monoclonal antihuman CD45 antibody (Dynabeads pan mouse IgG; Invitrogen). The plastic tube containing the mixture was placed next to a fixed magnet and the unlabeled granulosa cells collected. All samples were frozen at −80°C.

Approval for the use of granulosa cells was obtained from the Institutional Ethics Committees of Consejo Superior de Investigaciones Cientificas and Hospital Virgen Macarena (Seville, Spain), and every patient gave informed written consent.

Analytic Determinations

After 8 hours of fasting, blood was obtained for measurement of serum lipids and glucose in the discovery cohort (cohort 1). Serum glucose concentrations were measured in duplicate by means of the glucose oxidase method with the use of a Beckman Glucose Analyzer II. We used a Roche Hitachi Cobas c711 instrument to do the determinations. Total serum cholesterol was measured by means of an enzymatic colorimetric method through the cholesterol esterase/cholesterol oxidase/peroxidase reaction (Cobas Chol2). High-density lipoprotein cholesterol was quantified by means of a homogeneous enzymatic colorimetric assay through the cholesterol esterase/cholesterol oxidase/peroxidase reaction (Cobas HDLC3). Total serum triglycerides were measured by means of an enzymatic colorimetric method with the use of glycerol phosphate oxidase and peroxidase (Cobas Trigl). Low-density lipoprotein (LDL) cholesterol was calculated with the use of the Friedewald formula. Serum iron also was determined on a Roche Hitachi Cobas c711 by means of a colorimetric assay.

Serum ferritin was determined in patients from the discovery cohort with the use of a microparticle enzyme immunoassay (Axsym; Abbot Laboratories), with an intra- and interassay coefficient of variation <6%. Circulating hepcidin levels in serum were measured by a solid-phase ELISA (DRG Hepcidin 25 [Bioactive], EIA-5258; DRG International). Detection limit was 0.35 ng/mL. Intra- and