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A methodological and functional proteomic approach of human follicular fluid *en route* for oocyte quality evaluation☆

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ABSTRACT

Human follicular fluid (HFF) has been proven to contain biologically active molecules and proteins that may affect follicle growth and oocyte fertilization. Based on this concept, HFF proteomic characterization is having a significant impact in the delineation of a biomarkers' profile for oocyte quality estimation and, maybe, for in vitro fertilization (IVF) success improvement.

Follicular fluid is characterized by a vast protein complexity and a broad dynamic range of protein abundances that hinder its analysis. In this study we determined a proper solubilization and resolution method of HFF in 2-DE, minimizing sample manipulation, protein loss, and experimental artifacts. According to our methodology some low-abundance proteins were detected and identified by MS. Identified proteins were then functionally cross-linked by a pathway analysis. The generated path highlighted the occurrence in HFF of a tight functional-network in which effectors and inhibitors control and balance a space- and time-dependent induction/inhibition of inflammation, coagulation, and ECM degradation/remodeling. Such fine modulation of enzymatic activities exerts a fundamental role in follicle development and in oocyte competence acquiring. Alpha-1-antitrypsin resulted in the core protein of the delineated net and we interestingly detected its differential incidence in FF and serum from two small cohorts of patients who underwent IVF.

Biological significance

Human ovarian follicular fluid (HFF) is the in vivo microenvironment for oocyte during folliculogenesis. It contains biologically active molecules that may affect oocyte quality, fertilization, and embryo development. HFF is also one of the most abundant "waste product" in assisted reproduction. This makes HFF a readily accessible source of biomolecules for competence evaluation of collected oocytes. The methodological improvement we obtained in proteomics characterization of HFF lead to a wide overview

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on the functional correlation existing between several fluid components and on how their aberrant occurrence/activity may affect oocyte quality and ovulation.
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1. Introduction

The human ovarian follicular fluid (HFF) prevalently results from granulosa and theca cell secretion and from capillary diffusion [1–5]. During folliculogenesis, the blood-follicle barrier becomes more permeable to plasma molecular components and the follicular fluid (FF) acquires a consistent similarity to the serum [1,5]. Reflecting granulosa and theca metabolic activity as well as changing in follicle permeability, FF biochemical composition may reveal not only the stage of follicular development but also the general functional state of the follicle itself and the health condition of the generating organism. Moreover, variations in concentration of follicular fluid components can also affect the oocyte quality [6,7]. Actually, the FF is the microenvironment in which the oocyte develops and undergoes maturation, and it has been reasonably thought, and to some extent proven, to affect oocyte quality, fertilization and, maybe, embryo development [8].

Being aspirated with female gametes during oocyte recovery in in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) procedures, follicular fluid represents an abundant and easily-available biological material for follicle and oocyte quality investigations, without affecting the collected precious oocytes. As a matter of fact, being a probable source of biomarkers for oocyte/embryo quality and competence evaluation, the biomolecular characterization of HFF has attracted considerable attention. Actually, as a consequence of legal and ethical problems related to the over production of embryos and to their morphological/genetic selection, the oocyte and embryo quality assessment has become one of the major aims in reproductive biomedicine. At present, oocyte and embryo selection is prevalently attained using morphological criteria [9–14]. Nevertheless, the morphologic assessment is generally considered controversial and unsatisfactory, and, due to cumulus and corona cell presence, it can only be properly applied in ICSI procedure where the sperm injection occurs in "naked" oocytes [12,15]. Despite the biomedical urging of its functional characterization, FF protein composition as well as its role in follicular growth and oocyte maturation still remain to be clarified. Follicular fluid, like plasma, is actually characterized by a vast protein complexity and a very broad dynamic range of protein abundances that hinder its analysis. In the last years a number of proteomics attempts to determine the HFF protein composition have been performed but several of them show consistent sample treatment and/or a number of limitations in protein recovery, resolution, visualization and identification, as well as in protein isoform detection [4,6,16–28]. In order to obtain an overall proteomic snapshot of the fluid minimizing sample manipulation and experimental artifacts and increasing the amount of detectable protein isoforms and species, we tried to set up a proper solubilization and resolution method of HFF for 2-DE analysis. In spite of all its technical limits and the existence of alternative successful experimental methodologies developed to overcome such limits, 2-DE is in fact still retained, according to its high potential in protein resolution and in simultaneous visualization of hundreds of proteins, a powerful tool in investigations of complex protein mixtures, also towards biomarker discovery [29,30].

Here we proposed and compared two different protocols for sample preparation and three different modalities for sample loading in the first dimension. No prefractionation process was performed, nor depletion of the most-abundant proteins was applied. Even if the less-abundant protein detection may be affected by overlooking proteins, such as albumin or α-macroglobulin, we opted for the most direct analytical-way with the minimum of sample treatment and, consequently, with a *bona fide* reduction in the occurrence of methodological artifacts. Moreover, some of the most abundant serum proteins are retained to play specific roles in follicle and oocyte development and maturation. Consequently, their removal may limit functional characterization of the FF.

Using a denaturation/solubilization process by heating samples in a SDS/DTE solution and then performing protein precipitation and anodic cup-loading, very high quality 2-D gels were obtained. This allowed to properly select and excise protein spots to be analyzed by mass spectrometry. Numerous protein spots were thus unambiguously identified and, worthy of note, also some spots localizing at low molecular weight, that were excluded by the list of identified proteins in previously FF 2-D investigations, were successfully identified. Afterwards, to functionally correlate the identified proteins, a pathway analysis was performed by the MetaCore program and the built paths suggested the presence in mature follicles of a fine and tight control in enzymatic proteolysis that induces, regulates and/or inhibits inflammatory reaction, wounding response, coagulation cascade, and extracellular matrix (ECM) degradation/remodeling. According to scientific literature, the biochemical activities of identified proteins described, as expected, a functional environment characteristic of the ending phase of follicle maturation and ovulation. Based on the identified proteins and on the designation of functional hubs in the most significant network, albumin and alpha-1-antitrypsin were immunostained in FF and in serum from the same patients to evaluate correlation existing between the serum and the follicular pattern of these proteins.

2. Materials and methods

2.1. Follicular fluid and serum collection

Patients undergoing controlled ovarian hyperstimulation for in vitro fertilization were recruited for the study at the Centre for Diagnosis and Treatment of Couple Sterility, Institute of Obstetrics and Gynaecology, "Le Scotte" General Hospital in Siena. Human follicular fluid samples were obtained from 6 normo-ovulatory young women, 25–32 years old, and used for