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TECHNICAL INNOVATIONS

Secretome profile of mouse oocytes after activation using mass spectrum

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Abstract
Objective Mammalian oocytes undergo a cortical reaction after fertilization, releasing cortical granules and other proteins into the perivitelline space and inhibiting polyspermy. Few studies have evaluated the biological functions and properties of these proteins.
Study design We investigated mouse oocytes in which the zona pellucida (ZP) was present (ZP-intact group) or absent (ZP-free group).
Results After being activated by Srcl2, secreted proteins are collected from mouse oocytes. Mass spectrometry analysis was performed that identified proteins such as Ldhb, PADi6, Uchl1, Pebp1, Alb, Hsp90aa1, Prss1, trypsinogen 7, trypsin 4, trypsin 10, Sod1, Zp1, Zp2, Zp3, Akap8, Npm2, Pkm2 and Ppia in the ZP-free group. Proteins such as Ldhb, Uchl1, Prss1, trypsin 10, trypsinogen 7, and Ast1 were identified in the ZP-intact groups. The expression of some proteins, including Ldhb, Alb and Sod1, were initially detected following oocyte activation. The finding of four trypsin subtypes, such as Prss1, further support previous observations. Studies investigating the physiological functions and properties of these proteins are ongoing.
Conclusions Research on these cortical proteins provides a theoretical basis for understanding polyspermy inhibition at the level of ZP and gives technological support for fertilization detection, assessment of oocyte quality and embryonic culture.

Keywords Oocyte · Secretome · Activation · Mass spectrometry

Capsule This study provides a theoretical basis for understanding polyspermy inhibition at the level of the zona pellucida (ZP) and gives technical support for fertilization detection, assessment of oocyte quality, and embryonic culture.

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Introduction

Why does an oocyte only fuse with a single sperm? A central reason is that following penetration by spermatozoa, the oocyte undergoes a cortical reaction involving the release of cortical granules and other proteins into the perivitelline space. These cortical granules modulate the structure of the zona pellucida (ZP) to block polyspermy [1]. Studies have shown secretion of tissue plasminogen activator (tPA) [2], syntaxin [3], peptidylarginine deiminase (PAD) [4], ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) [5] and N-acetylglucosaminidase [6] following the activation of oocytes, however, these compounds cannot cause a reaction in the ZP. The addition of trypsin inhibitors into the culture medium can significantly elevate the amount of spermatozoa that penetrate the ZP [7], suggesting the existence of trypsin-like proteins among the secreted materials following oocyte activation. However, evidence supporting the hypothesis that oocytes release trypsin-like proteins is lacking. To date, the lack of information regarding the protein composition of cortical granules is one of the major obstacles inhibiting our understanding of the molecular mechanisms

underlying reproductive processes such as the blocking of polyspermy by ZP reactions [8].

The process of releasing cortical granules and other proteins during cortical reactions in oocytes is the single most significant secretion phenomenon that occurs from the beginning of gamete transformation to the embryonic stage until implantation. Identification of these secreted proteins and analysis of their biological and physiological functions could help elucidate the process of embryonic development and provide data to support the innovation of clinically assisted reproduction techniques. It is well known that the cortical reaction is a complex process. To date, the total number of mammalian cortical granule proteins remains unknown. However, several proteins have been identified in mammalian cortical granules using cytochemical techniques. These proteins include p62 and p56, which appear to be immunologically related to: sea urchin hyaline [9], ovoperoxidase [10], n-acetylglucosaminidase [6], calreticulin [11], p75 [12], and p32 [13]. With the development of microtechniques, experimental and immunohistochemical work has been performed on cortical granule proteins, leading to significant advances regarding cortical granule ultrastructure. However, these techniques have not allowed for biochemical or functional characterisation of these granules. Furthermore, these methods have been unable to classify secreted proteins, especially unknown secreted proteins, with similar two-dimensional structure or size. Therefore, building and utilising novel techniques to identify the physico-chemical properties and biological functions of unknown secreted proteins is of great interest.

Protein mass spectrometry is the dominant approach for identifying macromolecules, especially proteins, and plays a key role in research on protein structure. In particular, ionisation is a powerful technique for the detection of macromolecules with high molecular weight and low concentrations. The latest developments in protein mass spectrum technology have allowed for the detection of proteins at the picogram (pg) level and have enabled the monitoring of small amounts of protein (as little as 10 pg) under numerous physiological and pathological conditions. To date, very few studies have used mass spectrum methods to examine the roles of secreted proteins following oocyte activation. Here, we focused on the detection and analysis of secreted proteins after oocyte activation using liquid chromatography-MS/mass spectrometry (LC-MS/MS), with the goal of evaluating the relative integrity of the mass chromatograms of oocyte-secreted proteins (at least the proteins that are abundantly secreted). The identification, molecular characterisation and functional elucidation of these secreted proteins will provide insights into the regulation of fertilization and early embryonic development and may provide a theoretical basis for the assessment of oocyte quality in a clinical setting and the culturing of quality embryos.

Materials and methods

Preparation of animals and oocytes

Animal experiments were performed in accordance with the guidelines for animal research from the National Institute of Health and were approved by the Committee of Animal Ethics at the School of Medicine, Shanghai Jiao Tong University. Six-week-old female mice were chosen from the first hybridisation generation of C57BL/6 female mice and DBA/2 male mice and were housed under controlled environmental conditions (temperature=20–22 °C, humidity=50–70 %, 12-h light/12-h dark cycle). The animals received food and water ad libitum. A total of 5–10 IU of pregnant mare serum gonadotropin (PMSG) was used to cause superovulation in the mice, and after 48 h, mice were given 5–10 IU of human chorionic gonadotropin (HCG) intraperitoneally to induce ovulation. The animals were then sacrificed by decapitation 14 h after HCG treatment. The oviducts were snipped, the duodenal ampulla was opened with a needle tip and the cumulus-oocyte complexes were extracted and placed into N-2-hydroxyethylpiperazine-N0-2-ethanesulfonic acid buffer (HEPES) supplemented with 10 % albumen (Sigma, Milan, Italy). Unless otherwise specified, all chemicals and reagents were purchased from Sigma (St. Louis, MO).

Granulosa cells were removed using 150 IU/mL hyaluronidase with a glass tube with a 150 μm aperture. Cells were split into two groups, a ZP-free and a ZP-intact group. The ZP-free cells were soaked in Tyrode's acid for 10 s to remove the ZP and then washed five times in bacterial culture dishes. Both groups of cell groups were then washed 15 times in bacterial culture dishes using Dulbecco's phosphate-buffered saline (Invitrogen) supplemented with 3 % polyvinylpyrrolidone (PVP) and 1.0 % sodium pyruvate.

Activation of oocytes and collection of secretion proteins

Microdrops including 10 mM $SrCl_2$ and DPBS, PVP and sodium pyruvate (see above) were added to the bacterial culture dishes. The washed oocytes were then placed in the microdrops and activated for 1 h until most of the oocytes had exuded the second polar body. The microdrop solutions were collected into EP tubes (Axygen) and centrifuged at 1000 × *g* for 10 min. The supernatant was then collected and stored in ice boxes. $SrCl_2$ microdrop solution was used as a control. The samples were then submitted to LC-MS/MS analysis.

LC-MS/MS analysis

A Surveyor™ liquid chromatography system (ThermoFinnigan, San Jose, CA) consisting of a degasser, an MS Pump and an autosampler and equipped with a C 18 reversed phase column (RP, 150 μm×120 mm, custom packaging) was used. The pump flow rate was split 1:120 to achieve a column flow rate of 1.5 μL/min. The samples were loaded onto the column with a mobile-phase gradient from 5 % F (water with 0.1 % formic acid) to 65 % F (acetonitrile with 0.1 % formic acid) over 150 min. The MS data were acquired on an LTQ linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with an electrospray interface operated in positive ion mode. The temperature of the heated capillary was set at 170 °C. A voltage of 3.5 kV applied to the ESI needle resulted in a distinct signal, and the normalised collision energy was 35.0 KV. The number of ions stored in the ion trap was regulated by the automatic gain control. Voltages across the capillary and the quadrupole lenses were tuned by an automated procedure to maximise the signal for the ion of interest. The mass spectrometer was set such that one full MS scan was followed by ten MS/MS scans on the 10 most intense ions from the MS spectrum with the following dynamic exclusion settings: repeat count 2, repeat duration 30 s and exclusion duration 90 s.

Data analysis

To identify proteins in the above samples, spectra from each fraction were searched with the MASCOT algorithm against the non-redundant mouse protein database from IPI. All output results were combined together using custom software (BuildSummary) to delete keratins and redundant data.

Results

The LC-MS/MS results from the ZP-free and ZP-intact groups are shown in Table 1. From the given data, we found that 18 types of protein were obtained from the mass chromatogram of 600 mouse oocytes in ZP-free cells, including lactate dehydrogenase B chain (Ldhb), protein-arginine deiminase type-6 (PADi6), ubiquitin carboxyl-terminal hydrolase isozyme L1 (Uchl1), phosphatidylethanolamine-binding protein 1 (Pebp1), serum albumin (Alb), heat shock protein (HSP) 90-alpha (Hsp90aa1), Prss1 protease (Prss1), trypsinogen 7 (trypsinogen7), trypsin 4 (trypsin4), Try10-like trypsinogen (trypsin10), superoxide dismutase [Cu-Zn] (Sod1), zona pellucida sperm-binding protein 1 (Zp1), zona pellucida sperm-binding protein 2 (Zp2), zona pellucida sperm-binding protein 3 (Zp3), A-kinase anchor protein 8 (Akap8), nucleoplasmin-2 (Npm2), isoform M2 of pyruvate kinase isozymes M1/M2 (Pkm2) and peptidyl-prolyl cis-trans isomerase (Ppia). Data regarding protein abundance are listed in Table 1. We found that the concentration of Prss1 was the most abundant of all proteins detected.

Compared to the ZP-free group, only 6 types of proteins were found in abundance in the mass chromatogram of 2500 mouse oocytes in the ZP-intact group, including Ldhb, Uchl1, Prss1, trypsin10, trypsinogen7 and isoform 2 of astacin-like metalloendopeptidase (Astl) (Table 1). In the control mass chromatogram, only a few common contaminates such as keratin were identified.

Discussion

The recent development of mass spectrometry-based proteomic techniques has provided the foundation for high throughput, cost-effective and highly sensitive methods for protein analysis. This technology allowed the culture media conditioned by mouse and human IVF embryos to be examined [14]. Recently, Beardsley et al. (2010) employed electrospray ionization (ESI) mass spectrometry (ESI-MS) to identify proteins secreted by the pre-implantation embryo in vitro [15]. The use of conventional HPLC coupled with ESI-MS analysis has allowed the convenient identification of protein peaks. This strategy allows for protein detection in the picograme and thus seems suited for the analysis of small amounts of protein released by activated oocytes. Here, we demonstrated the feasibility of employing MS to identify proteins secreted by activated oocytes in vitro and identified a range of targets that are likely involved in the process of cortical reaction.

The data shown here (Table 1) revealed that ESI–MS was a more sensitive tool for secretome detection. Using this method, a diverse yet variable secretome generated by the activated mouse oocytes in vitro was detected. This method identified a total of 18 putative proteins released by activated oocytes, and known contaminant ions (e.g. keratin) and autolysed trypsin peaks were excluded. The known primary biological functions of the protein signals detected by ESI-MS are listed in Table 2.

Uchl1 was first detected by immunohistochemical methods using antibodies [5], which can be secreted after oocyte activation. Uchl1 was abundantly expressed, and the inhibition of its biological function by antibodies could be responsible for polyspermy of cattle oocytes, the mechanism of which is likely involved in the inhibition of cortical granule migration [16], Of the proteins detected, the expression of PAD has been shown to be essential for the normal development of the embryo. PAD is a family of post-translational protein-modifying enzymes that deamidate arginine into citrulline in the presence of calcium. The biological functions of PADs are important to cytoskeletal and chromatin reorganisation [17, 18]. PAD, found here to be

Table 1 Protein signals detected by ESI-MS in ZP-intact and ZP-free cells

Protein name	Mr(Da)	References	Detected group	Average score	Peptide count	Protein abundance (%)
Lactate dehydrogenase B chain	36,571	IPI00229510	ZP	50±2	5	7.58
			ZP-free	52±2	11	4.1
Prss1 protease, serine, 1	26,134	IPI00130391	ZP	64±7	4	6.06
			ZP-free	60±7	75	27.99
Trypsin 10	26,220	IPI00719897	ZP	48±2	4	6.06
			ZP-free	46	1	0.37
Trypsinogen 7	26,421	IPI00131674	ZP	46±2	2	3.03
			ZP-free	49±2	5	1.87
Astacin-like metalloendopeptidase	45,152	IPI00283246	ZP	44	1	1.52
Ubiquitin carboxyl-terminal hydrolase isozyme L1	24,837	IPI00313962	ZP	55	1	1.52
		IPI00653686	ZP-free	50±2	7	2.61
Arginine deiminase type-6	76777	IPI00170156	ZP-free	53±2	12	4.48
Zona pellucida sperm-binding protein 3	14728	IPI00857813	ZP-free	63±3	4	1.49
Phosphatidylethanolamine-binding protein 1	20830	IPI00137730	ZP-free	57±1	3	1.12
Serum albumin	68691	IPI00131695	ZP-free	46±2	2	0.75
Heat shock protein HSP 90-alpha	84786	IPI00330804	ZP-free	58±1	2	0.75
Zona pellucida sperm-binding protein 2	80208	IPI00117161	ZP-free	47±3	2	0.75
Peptidyl-prolyl cis-trans isomerase	18313	IPI00554989	ZP-free	45±2	2	0.75
Trypsin 4	26273	IPI00128108	ZP-free	49	1	0.37
Superoxide dismutase [Cu-Zn]	15942	IPI00130589	ZP-free	43	1	0.37
Zona pellucida sperm-binding protein 1	68722	IPI00310464	ZP-free	51	1	0.37
A-kinase anchor protein 8	76293	IPI00321739	ZP-free	62	1	0.37
Nucleoplasmin-2	23309	IPI00330555	ZP-free	48	1	0.37
Isoform M2 of Pyruvate kinase isozymes M1/M2	57844	IPI00407130	ZP-free	46	1	0.37

Total protein count of the ZP-free group: 268; ZP-intact group: 66

Average score: the score of this protein given by mascot, showing the confidence of this identification, the higher the value, the more confident

Peptide count: how many times of a peptide have been identified in this sample

Protein abundance: the abundance of a protein component in this sample

released by the embryo, was abundantly expressed in cortical granules and can be detected in the perivitelline space and the peripheral egg membrane after oocyte activation [4]. Anti-PAD antibodies incubated with developing embryos (from zygote to blastocyst) perturbed development of these embryos [4], Our observation of PAD family protein expression following oocyte activation is consistent with previous findings.

The cellular redox status of the early embryo has been the focus of much research. Of the proteins released following oocyte activation, Ldhb is known to participate in the regulation of the cellular redox status. In addition, Ldhb is seen in higher concentration in mouse zygotes than in blastocysts [19].

Gwatkin et al. (1973) added trypsin inhibitors (soybean trypsin inhibitor and a kind of low molecular weight trypsin inhibitor, ρ-aminobenzamidine) into medium, and the amount of spermatozoa penetrating the ZP was largely increased after electrical activation of oocytes [7]. As a result, it was proposed that the existence of trypsin-like proteins among the proteins secreted influence the ZP leading to a structural change to block polyspermy.

Tawia (1992) showed that the addition of leupeptin, a serine protease inhibitor, into medium can inhibit the activity of trypsin and reversed the low fertility rate due to the effect of A23187 [20]. The emergence of the four kinds of trypsin subtypes in our mass chromatogram, including Prss1, trypsinogen7, trypsin4 and trypsin10 (in particular the high concentration of Prss1) revealed the direct involvement of these proteins in the occurrence of the ZP reaction and the block of polyspermy. The presence of Alb was also found in the mass chromatogram. Direct evidence has been demonstrated supporting the suppression effect of Alb on the function of trypsin in certain amphibious animals [21], however, a role for Alb in protecting the egg membrane from self-trypsinisation and maintaining normal structure and function of the egg membrane remains to be elucidated.

Table 2 Common and Gene names, and main known functions of the protein signals detected by ESI mass spectrometry

Protein name	Common name	Gene name	Main function, where known
L-lactate dehydrogenase B chain	LDH-2	Ldhb	L-lactate dehydrogenase activity. Catalyses reaction: (S)-lactate + NAD (+) = pyruvate + NADH.
Protein-arginine deiminase type-6	ePAD	Padi6	Catalyses the deimination of arginine residues of proteins By similarity. May be involved in cytoskeletal reorganisation in the egg and early embryo.
Ubiquitin carboxyl-terminal hydrolase isozyme L1	PGP 9.5	Uchl1	Involves both in the processing of ubiquitin precursors and of ubiquitinated proteins; recognises and hydrolyses a peptide bond at the C-terminal glycine of ubiquitin; binds to free monoubiquitin and may prevent its degradation in lysosomes; may have ATP-independent ubiquitin ligase activity.
Phosphatidylethanolamine-binding protein 1	HCNPpp	Pebp1	Binds ATP, opioids and phosphatidylethanolamine. Serine protease inhibitor. may be involved in the function of the presynaptic cholinergic neurons of the central nervous system
Serum albumin	AFM	Alb	Regulation of the colloidal osmotic pressure of blood. Major zinc transporter in plasma,
Heat shock protein HSP 90-alpha	HSP90	Hsp90aa1	A stress actvated chaperonin with ATPase activity. Many cellular functions and targets.
Protease serine 1	TRYP1	Prss1	Serine-type endopeptidase activity
Trypsinogen 7	2210010C04Rik	trypsinogen7	Serine-type endopeptidase activity
Trypsin-4	Trypsin IV	trypsin4	Serine-type endopeptidase activity; metal ion binding
Trypsin-10	Trypsin X	trypsin10	Serine-type endopeptidase activity
Superoxide dismutase [Cu-Zn]	SOD1	Sod1	Destroys radicals which are normally produced within the cells and which are toxic to biological systems.
Zona pellucida sperm-binding protein 1	Zp-1	Zp1	Mediates species-specific sperm binding, induction of the acrosome reaction and prevents post-fertilization polyspermy
Zona pellucida sperm-binding protein 2	ZPA	Zp2	As above
Zona pellucida sperm-binding protein 3	Zpc	Zp3	As above
A-kinase anchor protein 8	AKAP95	Akap8	Mediates the subcellular compartmentation of cAMP-dependent protein kinase (PKA type II).
Nucleoplasmin-2	NPM2	Npm2	Probably involved in sperm DNA decondensation during fertilization.
Pyruvate kinase isozymes M1/M2	Pyruvate kinase 2/3	Pkm2	Catalyses the transfer of a phosphoryl group from phosphoenolpyruvate (PEP) to ADP, generating ATP. Stimulates POU5F1-mediated transcriptional activation
Peptidyl-prolyl cis-trans isomerase A	Cyclophilin A	Ppia	Catalyses the cis-trans isomerisation of proline imidic peptide bonds in oligopeptides, accelerates the folding of proteins.
Astacin-like metalloendopeptidase	Oocyte astacin	Astl	Metalloendopeptidase activity. zinc ion binding

Ovoperoxidase was found in sea urchin eggs and was also detected in the cortical granules of unfertilized mouse oocytes at the ultrastructural level using the 3,3′-diaminobenzidine (DAB) [10]. Following artificial activation, ovoperoxidase was found to be present on the oocyte surface, in the perivitelline space, and in the ZP. Following fertilization, the enzyme was inferred to harden the ZP, as both peroxidase inhibitors and tyrosine analogs prevented hardening [22]. In addition, ovoperoxidase was demonstrated to be associated with a slow block to polyspermy. Given that the product of Sod1 was simply the substrate of ovoperoxidase, the existence of Sod1 in the mass chromatogram suggests that the protective effect of Sod1 on the egg membrane acts against the attack of free radicals and promotes ZP hardening to prevent polyspermy.

We found that either the kinds or the amounts of protein from the ZP-free groups were largely exceeded compared to ZP-intact groups, which is most likely related to the distribution of the secreted oocyte proteins. Most of these proteins were located in the perivitelline space, whereas only a few of them diffused into medium across the ZP. When necessary, we assessed the diffusion of these proteins by detecting the kind and amount of secreted oocyte proteins in the medium surrounding the oocytes. Chen et al. (2003) observed the extrusion of the secondary polar body 6 h after fertilization of human ovum to judge whether fertilization occurs [23], and intracytoplasmic sperm injection (ICSI) was carried out for the non-fertilized

oocytes. According to our mass chromatogram, we can detected the existence of proteins in the medium surrounding human oocytes (such as Prss1) to assess the fertilization of the oocyte as early as possible. These results provide the technological means and supporting data that will allow clinical laboratories to apply ICSI in an earlier period.

As early as 2002, Fuzzi et al. [24] suggested that by detecting the status of sHLA-G secreted by the embryo, the embryonic quality could be assessed. Rebmann (2010) described the sHLA-G index by evaluating the work of various research institutes in German [25]. In 2008, Borgatti [26] proposed an approach to identify oocyte quality via detecting the sICAM-1 secreted by oocytes. Whether the difference in the protein types or amounts secreted by different oocytes observed from mass chromatography (e.g. the amount of UCH-L1 secreted) can be used as an index of oocyte quality remains to be elucidated. If this MS-based index is possible, it may be possible to use it clinically for embryo selection.

The composition of the perivitelline space provides an important environment for embryonic development. P62 and P56 have been identified as cortical granule proteins and can form cortical granule amicula after secretion. The inhibition of their function can affect the cleavage of oocytes and embryos [27]. In addition to the experiments described here, 650 vitrificated Kunming mouse eggs were thawed and perforated on the ZP using diode laser-assisted hatching. The obtained mass chromatogram of the secreted proteins after activation only showed expression of Prss1 and trypsinogen7 (data not shown). It is likely that these proteins found in the perivitelline space were missed during the freeze-thaw cycle. Further experiments are needed to verify whether the addition of some proteins found in the mass chromatogram into the perivitelline space could help with embryo development (especially the development of frozen oocytes).

The protein mass spectrum technique has been the primary method for macromolecule identification, especially in the research of protein structure and properties. To date, the detection range of this technique has reached to within 10 pg, and one oocyte at one point in time can secrete approximately 20 types of proteins, totaling between 50–100 pg [28]. Thus, the protein content secreted by 500–5000 oocytes is sufficient for the detection of abundant proteins.

In this study, we used mass spectrum to detect secreted proteins following oocyte activation. Among the proteins detected following oocyte activation, Ldhb, Alb, Sod1 were found. The detection of UCH-L1 and PAD were consistent with previous studies. Furthermore, the existence of the four trypsin subtypes, including Prss1, confirms previous proposals. However, the exact biological functions of these secreted proteins remains unclear, and a detailed functional analysis is required to assess whether their release by oocytes is an important property of cortical reactions. In the future, we plan to use a CB100 (Cell Biosciences) iso-electric focusing (IEF) microiontophoresis for the identification and investigation of these secreted human oocyte proteins [29] because the precision of IEF microiontophoresis allows detection of signals as low as the signal protein content secreted by 25 cells.

Conclusions

Research on these cortical proteins provides a theoretical basis for understanding polyspermy inhibition at the level of zp and gives technological support for fertilization detection, assessment of oocyte quality and embryonic culture.

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Conflict of interests The authors declare that they have no conflicts of interest.

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