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Global proteome profiling of cumulus free immature buffalo (*Bubalus bubalis*) oocytes

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Abstract
Global proteome profile of immature buffalo oocytes (cumulus free, zona pellucida enclosed) was done and 1662 proteins were identified (FDR <1%) from isolated crude proteins. Among these proteins characterized and uncharacterized proteins were obtained. The data (no. of proteins) obtained was compared with evidenced data of other buffalo oocytes. Among the identified proteins, oocyte germ cell potential markers and RNPs associated proteins were found. Our finding with Bioinformatics analysis suggests, enriched pathways associated with immature oocyte. Our findings provide comprehensive catalogue of proteins according to biological process, molecular function and cellular component through PANTHER analysis. For pathway analysis KEGG tool was used and abundance of protein was classified in different pathways. Here we have discussed notable proteins obtained in our profile which have crucial significance towards oocyte quality and probable marker associated properties. The proteome profile of immature oocyte crude proteins found in this study can be source for further developmental studies.

Keywords: Immature oocyte, proteome, profiling, in-solution and mass spectrometry

1. Introduction
There are many biomolecules in mammalian oocyte, including proteins, peptides, hormones and metabolites. These biomolecules either get stored up to certain time within oocyte or degraded under limits, but plays key role in specific stages of oocyte development. Immature oocytes of mammals contain mRNA and protein complexes which are formed during oogenesis. These mRNA and proteins constitute the developmental program to get oocyte competent. Proteomic profiling provides new avenues for oocyte function also to unravel the complexities between the protein levels and corresponding transcripts, which are more or less still to be, deciphered (De Sousa *et al.*, 2009) [4]. Differences in proteome profile often seen since oocyte sustain silent mRNA, which gets activated only after fertilization (Tadros *et al.*, 2009) [15]. Also, many genes expressed in mammalian (mouse and bovine) oocyte tend to remain conserved (Lotan *et al.*, 2014) [11]. In a recent research, Chen *et al.*, 2016 [3] examined the proteomics of various maturation stages and developmental competence status of buffalo oocytes. These authors investigated the protein profile and identified a total of 3,763 proteins of buffalo oocytes. Proteome of mammalian species are being compared but many facets still to be uncovered. Since, after the resumption of meiosis numerous events occurs in nucleus as well as in cytoplasm which are called nuclear and cytoplasmic maturation respectively. This interlinked event of coordinated remodelling and repositioning inside nucleus and cytoplasm remains continued to complete maturation. During oocyte maturation, synthesis and degradation of specific proteins occurs in highly regulated manner. For instance, receptors for hormones (LHr, FSHr) are formed (Calder *et al.*, 2003) [2] to prepare oocyte for cell signalling and associated changes. Considerable changes in ribosomal, mitochondrial, endoplasmic reticulum, golgi body, cytoskeletal proteins and antioxidant system happens during cytoplasmic maturation (Ferreira *et al.*, 2009) [6]. These events changes set of proteins from those of immature stage. In view of this, our study was carried out with the aim that buffalo immature oocyte maternal proteins repertoire can be explored. Further, from these proteins set, factor proteins which may be responsible for developmental competence can be identified. However, there is interest in isolating oocyte proteins through different buffer systems and also identification through different high throughput proteomic techniques, so that complete repertoire of oocyte proteins can be deduced. This is the first report of crude proteins set proteome of buffalo where acetone precipitation or clean up step was not used. Present study, will provide a reference for future studies in biomarker discovery linked to development and associated metabolic events, which will enhance the efficacy of *in vitro* protocols.

2. Material and Methods

2.1 Extraction of oocyte proteins

Buffalo ovaries were obtained from slaughter house (Ghazipur, New Delhi) in antibiotics-fortified warm saline (32-38 °C) with streptomycin (500 µg/ml). After washing of ovaries, oocytes were aspirated in aspiration medium along with follicular fluid and maintained at 37 °C. Morphologically immature oocytes (cumulus oocyte complex) were separated under zoom stereo microscope. Further oocytes were denuded using 0.75mg/ml hyaluronidase enzyme. To ensure complete removal of any residual cumulus cells, oocytes were washed three times with Ca^{++} and Mg^{++} free DPBS medium. To get rid of any protein degradation 1% v/v protease inhibitor cocktail was used and sample was cryopreserved (-80 °C) in Ca^{++} and Mg^{++} free DPBS till further use. Pooled immature oocytes were subjected to 200 µl of cell lysis buffer (2% SDS, 1% v/v protease inhibitor cocktail) for 30 min on ice followed by sonication (30% amplitude for four cycles 30s on 5s off pulse). The samples were centrifuged (12000×g at 4 °C for 10 min), and the supernatant was collected. Protein estimation was performed using Bradford method (Bradford. 1976) [1].

2.2 Tryptic digestion and fractionation

Overnight treatment with trypsin (1:20) at 37 °C was used to digest the measured protein. Subsequently, MilliQ® water stopped the reaction, after which the peptides were vacuum-dried using SpeedVac and stored until further use at -80 °C. Samples were loaded onto C18 column (4.6 x 250mm, 5µm, Grace) on a Dionex, Quaternary UHPLC system (Ultimate 3000, Thermo Fisher). The HPLC solvents used included Solvent A, which was 10 mM triethylammonium bicarbonate (TEAB) and Solvent B which was 10 mM TEAB in 90% acetonitrile (ACN). Using a continuous gradient elution method (95-100 percent ACN) over a duration of 81 min, the peptides were fractionated at 25 °C with a flow rate of 1 ml/min. The gradient was set up as follows: 0 to 2% B for 5 min, 2 to 60% B for 60 min, 60 to 100% B for 10 min, held the same gradient for next 1 min followed by 2% B for 5 min. By combining most hydrophobic with most hydrophilic peptides based on the concatenation method, a total of 96 time-based fractions were obtained and further pooled into 12 individual fractions. Then the pooled fractions were lyophilized, acidified with 0.1% formic acid and desalinated with C18 Zip tips (Merck). For LC-MS/MS analysis, the eluted peptides were lyophilized and stored at -80 °C.

2.3 Desalting of peptides

The pooled fractions were reconstituted in 0.1% formic acid 30µl and subjected to desalination using the column Zip Tip C18. For the effective binding of peptides in the C18 column, the Zip Tip C18 column was equilibrated with 100% ACN followed by 0.1% formic acid and then repeated pipetting with digested samples was performed. Finally, to extract the unbound peptides from the column, the column was washed with 0.1 percent formic acid. The bound peptides were eluted with buffer containing 60% ACN and 0.1% formic acid in ratio of 1:1. The eluted peptides were lyophilized using the Speed VAC concentrator and stored at -80ºC till further analysis.

2.4 Mass spectrometry analysis

The digested peptides were reconstituted in 0.1% formic acid in LCMS grade water and subjected to nano-LC (Nano-Advance, Bruker, Germany) followed by identification by captive spray-Maxis-HD qTOF (Bruker, Germany) mass spectrometer (MS) with high mass accuracy and sensitivity. The peptides were enriched in nano-trap column (Bruker Magic C18AQ, particle size-5 µm, pore size-200 Å) and eluted on to analytical column (Bruker Magic C18AQ, 0.1 × 150mm, 3 µm particle size and 200 Å pore size) using linear gradient of 5-45% acetonitrile at 800 nl/min over 55min. Positive ions were generated by electro spray and the QTOF operated in data dependent acquisition mode. A TOF MS survey scan was acquired (m/z range of 400–1400) and 6 most intense were sequentially selected by Q1 for MS-MS analysis.

2.5 Data analysis

Otof control has produced peak lists using the Hystar post processing program to automatically subtract baseline. The search requirements for Mascot MS/MS ion is as follows: taxonomy other mammalia, trypsin digestion, allowing up to one missed cleavage, variable modification oxidation of methionine, fixed modification cysteine as carboxyamidomethylation or propionamide, peptide tolerance of 50 ppm, and MS/MS tolerance of 0.05 Da. The "ion score cutoff" was manually adjusted to 15, thereby excluding matches of the lowest quality. 1 % FDR was introduced at both protein and peptide levels to remove false positives. The gene ontology of proteins identified has been derived from PANTHER (protein analysis through evolutionary relationship) http://www.pantherdb.org/ database, covering biological processes, cellular components, and molecular functions. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/pathway.html) has been used for pathway study of identified proteins. CYTOSCAPE (https://cytoscape.org/) tool was used for visualization of interaction among identified proteins.

2.6 Statistical Analysis

For protein identification by MS, 1% FDR was applied at both peptide and protein levels to eliminate the false positives. GO and pathway enrichment analysis and Cytoscape was performed with $p \leq 0.05$ as the cut off criterion, and were considered to be statistically significant.

3. Results and Discussion

3.1Protein identification by LC-MS/MS

A Total of 2500 oocyte were used for recovery of proteins. 100 µg proteins were used for further MS analysis. A total of 1662 proteins were identified through Protein Scape software among which few proteins with high protein score found in our study is listed in Table 1. These proteins may be more stable or more abundant in comparison to those proteins, which were recovered while using other extraction buffer. Less number of proteins obtained may also be due to single stage of study (immature stage). During the preliminary experiments for the present study, we found that during acetone precipitation degradation of substantial amount of protein occurs. To get rid of this problem, we proceeded with crude proteins. Since crude protein was used, so in this case many interfering molecules (lipids carbohydrate salts and detergents) may reduce the net number and types of proteins obtained during C_{18} ziptip procedure.

Table 1: List of proteins with high protein score in immature buffalo oocyte

S. No.	Accession No.	Protein	Peptides	pI	MW [kDa]	Scores	SC [%]
1	tr|F1N757|F1N757_BOVIN	Uncharacterized protein OS=Bos taurus GN=TTN PE=4 SV=2	45	5.9	3811.5	1980.6 (M:1980.6)	0.9
2	tr|F1MGT1|F1MGT1_BOVIN	Uncharacterized protein (Fragment) OS=Bos taurus GN=SYNE1 PE=4 SV=2	14	5.4	1004.5	586.7 (M:586.7)	1.2
3	tr|A6QP77|A6QP77_BOVIN	TCOF1 protein (Fragment) OS=Bos taurus GN=TCOF1 PE=2 SV=1	12	9.2	138.8	421.0 (M:421.0)	4.4
4	tr|G3N1N7|G3N1N7_BOVIN	Uncharacterized protein OS=Bos taurus GN=MKI67 PE=4 SV=1	11	9.7	330.9	499.6 (M:499.6)	2.6
5	tr|F1N6H4|F1N6H4_BOVIN	Uncharacterized protein (Fragment) OS=Bos taurus GN=MACF1 PE=4 SV=2	11	5.2	824.2	368.6 (M:368.6)	0.9
6	tr|E1BGA4|E1BGA4_BOVIN	Histone-lysine N-methyltransferase OS=Bos taurus GN=ASH1L PE=4 SV=1	10	9.4	332.3	393.8 (M:393.8)	2.9
7	tr|F1N1S2|F1N1S2_BOVIN	Uncharacterized protein OS=Bos Taurus GN=MAP1B PE=4 SV=2	10	4.7	270.1	365.6 (M:365.6)	2.3
8	sp|G3N131|H11_BOVIN	Histone H1.1 OS=Bos taurus GN=HIST1H1A PE=1 SV=1	10	10.9	22.1	294.5 (M:294.5)	15.1
9	sp|Q9BH10|ZP2_BOVIN	Zona pellucida sperm-binding protein 2 OS=Bos taurus GN=ZP2 PE=1 SV=1	10	8.1	79.5	292.1 (M:292.1)	10.8
10	tr|G3MYT9|G3MYT9_BOVIN	Kinesin-like protein OS=Bos taurus PE=3 SV=1	10	5.4	202.9	204.1 (M:204.1)	1.4
11	tr|F1MHA1|F1MHA1_BOVIN	Uncharacterized protein (Fragment) OS=Bos taurus GN=KMT2A PE=4 SV=2	9	9.2	417.3	345.8 (M:345.8)	1.5
12	tr|A5D7E8|A5D7E8_BOVIN	PDIA3 protein OS=Bos taurus GN=PDIA3 PE=2 SV=1	9	6.4	56.9	328.2 (M:328.2)	15.6
13	tr|O11993|O11993_BVDV	Pestivirus type 1 cytopathic genomic RNA, complete genome OS=Bovine viral diarrhea virus PE=4 SV=1	9	9.1	558.5	286.1 (M:286.1)	1.5
14	tr|Q58DI4|Q58DI4_BOVIN	Zona pellucida glycoprotein 3 preproprotein OS=Bos taurus GN=ZP3 PE=2 SV=1	9	8	41.8	276.0 (M:276.0)	8.7
15	tr|G5E5D5|G5E5D5_BOVIN	Uncharacterized protein OS=Bos taurus GN=LOC787476 PE=4 SV=1	8	6.2	770.6	465.9 (M:465.9)	0.9
16	tr|F1MQI3|F1MQI3_BOVIN	Uncharacterized protein OS=Bos Taurus GN=NEB PE=4 SV=2	8	9.2	801.4	455.5 (M:455.5)	1.3
17	tr|F1MNN6|F1MNN6_BOVIN	Major vault protein OS=Bos taurus GN=MVP PE=4 SV=1	8	5.4	98.9	346.1 (M:346.1)	8.4
18	tr|J9U8U3|J9U8U3_BOVIN	Cardiomyopathy associated protein 3 (Fragment) OS=Bos taurus GN=CMYA3 PE=2 SV=1	8	5.9	377.1	304.3 (M:304.3)	2
19	tr|F1MUD3|F1MUD3_BOVIN	Uncharacterized protein OS=Bos taurus G N=SPEF2 PE=4 SV=2	8	5.4	209.4	293.9 (M:293.9)	1.4
20	tr|G3MWV5|G3MWV5_BOVIN	Uncharacterized protein OS=Bos taurus GN=HIST1H1E PE=3 SV=1	8	11	21.9	292.1 (M:292.1)	16.4
21	tr|F1MU48|F1MU48_BOVIN	DNA topoisomerase 2 (Fragment) OS=Bos taurus GN=TOP2B PE=3 SV=2	8	8.4	180.6	281.9 (M:281.9)	2.3
22	tr|F1N415|F1N415_BOVIN	Uncharacterized protein OS=Bos taurus GN=PCLO PE=4 SV=2	8	5.8	546.8	272.6 (M:272.6)	0.8
23	sp|Q76LV2|HS90A_BOVIN	Heat shock protein HSP 90-alpha OS=Bos taurus GN=HSP90AA1 PE=1 SV=3	8	4.9	84.7	256.1 (M:256.1)	8.2
24	tr|F1MH71|F1MH71_BOVIN	Uncharacterized protein OS=Bos taurus GN=PHF2 PE=4 SV=2	8	9.1	123.9	229.7 (M:229.7)	3.2
25	tr|B9X245|B9X245_BOVIN	Factor VIII OS=Bos taurus GN=F8 PE=2 SV=1	8	7.8	263.3	224.6 (M:224.6)	1.3
26	sp|E1B7Q7|TRIPC_BOVIN	E3 ubiquitin-protein ligase TRIP12 OS=Bos taurus GN=TRIP12 PE=2 SV=2	8	8.7	220.3	211.1 (M:211.1)	1.4
27	sp|Q0IIJ2|H10_BOVIN	Histone H1.0 OS=Bos taurus GN=H1F0 PE=2 SV=3	7	10.9	20.9	364.8 (M:364.8)	12.4
28	tr|E1BHT5|E1BHT5_BOVIN	Uncharacterized protein OS=Bos taurus GN=UBR4 PE=4 SV=1	7	5.7	572.2	330.8 (M:330.8)	0.8
29	tr|F1MRK2|F1MRK2_BOVIN	Uncharacterized protein (Fragment) OS=Bos taurus GN=SPEN PE=4 SV=2	7	7.4	402.3	293.7 (M:293.7)	2.2
30	tr|E1BN16|E1BN16_BOVIN	Uncharacterized protein OS=Bos taurus GN=CCDC18 PE=4 SV=2	7	5.5	169.9	243.1 (M:243.1)	2.6

3.2 Functional classification of identified proteins

Gene ontology of identified proteins was derived from PANTHER database. Most proteins were found to be of cellular part (45%) followed by organelle proteins (26%), the proteins of extra cellular matrix (1%) and cell junction (2%) were of less abundance, which can be correlated with abundances according to bidirectional communication between cumulus cells and oocyte. In case of molecular function related proteins, binding proteins (40%) and catalytic activity (36%) proteins were highly abundant compared to translation regulator activity (1%), receptor activity (5%), transporter activity (7%), channel regulator activity (1%), signal transducer activity (2%) and structural molecule activity (9%) proteins. For the biological process, many processes came into picture, where we found metabolic process (23%) and cellular process (30%) were dominant, fewer proteins were present in reproduction (1%) and biological adhesion (1%) related process. Normal cellular and metabolically active proteins (23%) were abundant. Notable protein content were related to developmental process (6%), response to stimulus (8%) and cellular component biogenesis (8%).

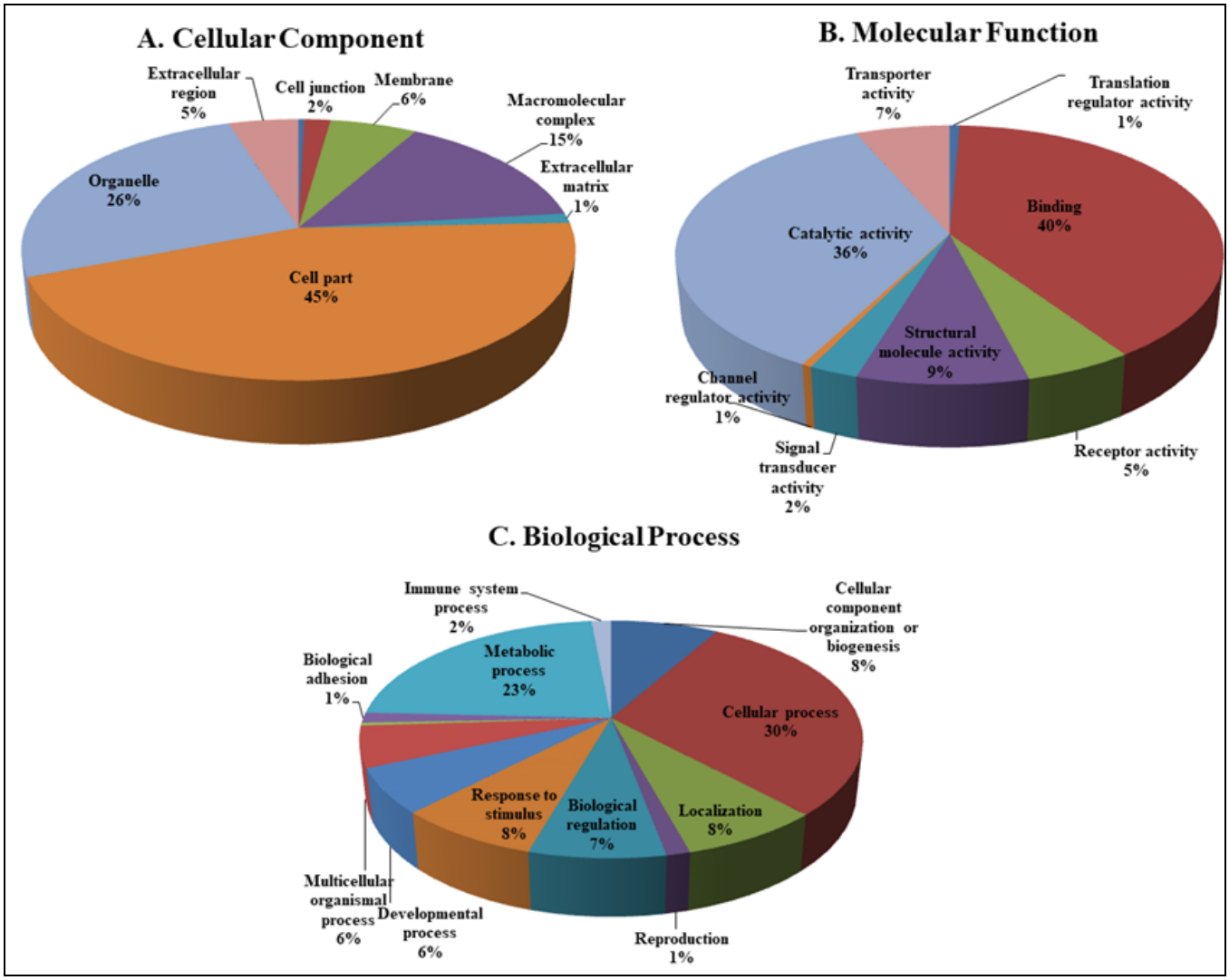

Fig 1: Gene ontology classification of proteins on the basis of their involvement in A. molecular function, B. biological process, and C. cellular component using PANTHER software.

3.3 KEGG pathway analysis of profiled proteins

We analyzed profiled proteins based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. We found that profiled proteins were mainly enriched in metabolic pathways, PI3K-Akt signaling pathway, Regulation of actin cytoskeleton, Endocytosis, Rap1 signaling pathway, Focal adhesion, MAPK signaling pathway, Ras signaling pathway, Protein processing in endoplasmic reticulum, RNA transport, Calcium signaling pathway and Oocyte meiosis; these signaling pathways may be associated with early embryonic development. Of note, we found that the number of proteins enriched in the metabolic pathway is the largest; the PI3K-Akt signaling pathways are also more active. Most enriched pathways (Table 2) and their associated function, revealed that, although oocytes are in growing phase but at halt where signalling pathways is highly active through biosynthesis of biomolecules essential for resumption of meiosis and completion of maturation process.

Table 2: Most enriched pathways in immature oocyte

S. No	Signaling pathways and number of proteins involved
1	Metabolic pathways - (87)
2	PI3K-Akt signaling pathway - (35)
3	Regulation of actin cytoskeleton - (29)
4	Endocytosis - (29)
5	Rap1 signaling pathway - (25)
6	Focal adhesion - (25)
7	MAPK signaling pathway - (23)
8	Ras signaling pathway - (20)
9	Protein processing in endoplasmic reticulum - (19)
10	RNA transport - (17)
11	Calcium signaling pathway - (16)
12	Phospholipase D signaling pathway - (16)
13	Oocyte meiosis - (16)
14	Insulin signaling pathway - (15)
15	Phosphatidylinositol signaling system - (15)

3.4 Construction of networks

To understand the biological significance of buffalo oocyte proteins, protein interaction network for profiled proteins was built by cytoscape software (Fig. 2). A majority of the proteins were grouped into a few major pathways. Among these cytoskeleton organization, chromosome and organelle organization pathways were prevalent, which represents these pathways are active at immature stage. These processes are interlinked since, during oocyte development cytoskeleton proteins plays important role in cellular movements where, events like organelle biogenesis and chromosomal organisation occurs.

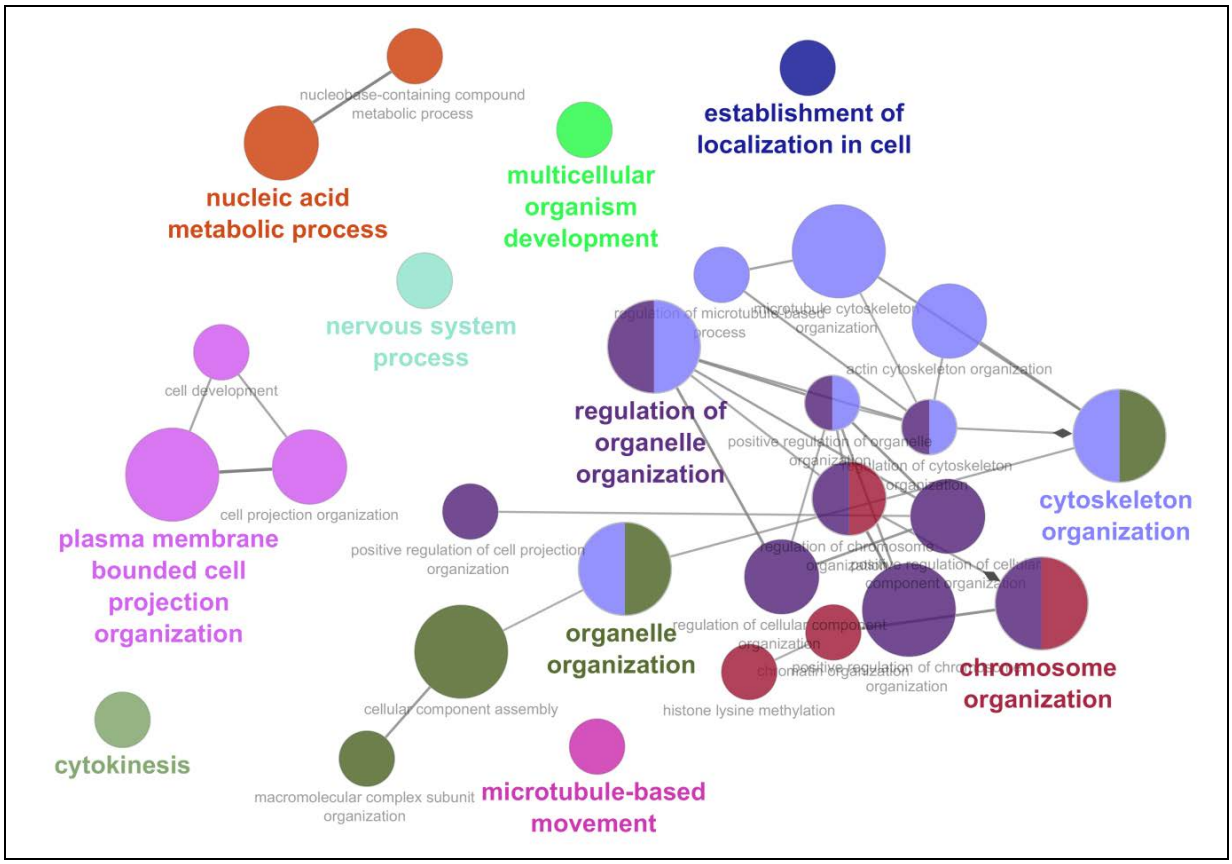

Fig 2: Network depicting the biological significance of proteins identified in cumulus free immature buffalo oocyte.

4. Biomarker potentials in buffalo oocyte

Current embryo research is seeking for new markers in mammalian oocyte for optimal results in *in vitro* production. Expression of proteins in oocyte gets altered in response to changes during different stages of maturation in mammals, which may serve as biomarkers against specific stage of development such as GV, MI, and MII. Although a number of oocyte proteins are reported as potential biomarkers in mammalian oocyte, still limited information is available in view of marker selection. Each stage of oocyte maturation is ideal to monitor complete set of protein repertoire which plays essential roles in oocyte development. Few groups have documented the identification of potential protein biomarkers in oocytes of domestic animals. Recently hspα 90 has been shown to effect meiotic maturation in pig oocyte through coupling with MAPK signalling pathway (Liu *et al.*, 2018) [10]. Here the researchers found that in presence of hspα 90 inhibitor (17 AAG) germinal vesicle breakdown got delayed. Thus its presence at immature stage is essential for progression of maturation. Yurttas *et al.*, 2010 [17] showed that Lipocalin (LCN1) appeared to be down regulated during maturation which suggest that it may have some role at specific stage of development in oocyte. From an economic viewpoint, one of the most crucial applications of new stage specific markers in buffalo oocytes would be to classify factors to validate oocyte developmental competence and quality. Different research groups across the world have used oocyte for detection of quality and competence factors in many species. For example, MPF plays a significant role in three ways; firstly it is triggered from prophase I arrest at GVBDD for the start of meiosis. Secondly, during the transfer of oocytes from MI to MII, where major chromosomal segregation events occur, MPF plays a crucial role. The third role of MPF is self-maintenance activities at MII arrest and programme for its destruction during egg activation (Jones, 2004) [9]. Previously the members of UCH-family have also been observed to express in mammalian oocytes (Ellederova *et al.*, 2004; Sekiguchi *et al.*, 2006; Mitango *et al.*, 2015) [5, 14, 12]. Few members of UCH-family also associate with meiotic spindle (Mitango *et al.*, 2015) [12]. The expression of Ubiquitin C-terminal hydrolases (UCHs) in immature stage of oocytes may play an important modulating function in development. It functions by removing multi-ubiquitin chains from proteins that are posttranslationally modified by ubiquitination. From some previous reports (Valdez *et al.*, 2004; Gonzales *et al.*, 2005; Sakai *et al.*, 2016; Gonzales *et al.*, 2005) [7, 8, 13, 16] it is evident that TCOF1 encodes nucleolarphosphoprotein Treacle (observed largely in nucleoli), which plays essential role in ribosomal DNA transcription, cell survival and ribosome biogenesis. Tcof1 may also be essential for cell defence against oxidative stress-induced cell death. Thus role of tcof1 in oocyte may provide an avenue for development, which ultimately influences the proliferation and proper differentiation during development of

particular embryonic cells. Importantly, identification of HSP90, LCN1, MPF, MAPK, UCHL1, PKB, NPM2, GST, PDE4D and PRKA in this study which control maturation and development, found to express in immature stage may also be invaluable and act as indirect marker of competence. In this study we found that most proteins were involved in cell communication and metabolism which may be important characteristics of immature oocyte in buffalo. Large quantity of oocyte is primary requirement for isolation of adequate amount of proteins and limitation in efficiency of technology used for investigation also affects total number of identified proteins.

5. Conclusion

In summary, this is the first high throughput proteomics analysis of crude proteins in immature cumulus free buffalo oocyte. In the present study, we identified 1662 proteins (FDR <1%) from isolated crude proteins of immature buffalo oocytes (cumulus free, zona pellucida enclosed). Although purification was not performed where some degree of proteins gets lost, however without purification some proteins may not be detected because of other cellular constituents. Germline proteome behaviour of immature oocyte which is about to receive signals signature to get mature can be explored. Complete set of maternal proteins at immature stage can be compared to other published data on buffalo in immature condition. Outcome of our findings suggests pathways associated with immature oocyte and provides important proteins related to developmental competence. Most of the proteins identified were associated with fundamental metabolic pathways or with cytoskeletal function. This study represents the first step in identification of crude protein map of buffalo oocyte.

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