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Exosome-Enriched Maternal Serum Proteins Contain Signals of Conceptus Growth and Estrous Cycle in Buffalo (Bubalus bubalis)

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Abstract

The membrane-bound extracellular vesicles (EVs) the exosomes, that carry proteins, nucleic acids, and lipids in their cargo from the cells of origin, play a significant role during pregnancy establishment in different species. This study was thus designed to understand whether the precipitated fraction of maternal serum proteins by commercial exosome isolation reagents helps to understand the cycle and early-pregnancy signals by two-dimensional electrophoresis and to identify the differentially expressed protein spots by LC-MS/MS mass spectrometry analysis for their involvement in the different functional pathways and networks. Serum exosome-enriched fraction were isolated from 0.5 mL serum of non-pregnant day 0, day 10, and day 15 of the cycle ($n = 6$), and early pregnancy days 15, day 30, and day 60 ($n = 5$) buffaloes. The protein in the precipitate were sonicated, and passed through Sephadex G25 spin columns, quantified, and analyzed by one- (1D) and two-dimensional (2D) gel electrophoresis. Differentially expressed proteins were analyzed to understand the cycle and pregnancy stage-specific changes. A total of 9 unique spots were analysed by mass spectrometry and identified by NCBI non-redundant protein database search. Results indicated exosome protein contents at cycle day 10 were significantly lower than the other days of the cycle and early pregnancy samples. The 1D electrophoresis did not reveal any difference but the 2D analysis revealed 8 pregnancy-specific and one cycle-specific protein spots that are significantly upregulated. Mass spectrometry analysis of these 9 spots identified 19 different proteins by database search. The proteins SLAM9 and MARK1 were identified in multiple spots. The proteins identified are indicated in the mitosis, cell cycle regulation, morphogenesis, and regulation of several cellular and molecular pathways relating to conceptus development.

In conclusion, the exosome-enriched maternal serum proteins contained signals of the cycle, early pregnancy, and conceptus growth. The proteins identified by this approach might be good targets for defining the critical period of pregnancy/cycle in buffaloes and future research on exosomes.

Keywords: Buffalo, early pregnancy, serum exosomes, exosome proteins, protein profile.

Introduction

In buffalo, identification of signals in the maternal circulation during the critical period of conception, the maternal recognition of pregnancy (MRP, days 15 – 17), and the implantation (days 21 – 60 of pregnancy) are important, as these events determine the fate of the cycle and pregnancy. Manipulation of these events might help to devise better reproductive management strategies.

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During MRP, the corpus luteum function that controls the length of the estrous cycle is retained by the release of interferon-*tau* (IFNτ) from the conceptus. The IFNτ that initiate the anti-luteolytic mechanism in the uterus, to ensure the progesterone support in the uterus which is essential for conceptus growth. It remains in the maternal system for a very short period of time and is present in a very minute quantity that skip detection even with highly sensitive Radio immune assay. The other important event that happens in buffalo and other ruminants is the implantation by the formation of synepitheliochorion. This process is initiated by the formation of binucleated trophoblastic cells as a result of acytokinetic mitosis. The binucleated cells, after formation, migrate to the direction of the mono nucleated caruncular epithelium and fuse to form trinucleated hybrid cells. The continued fusion of tri-nucleated cells in the cotyledon and caruncular junctions results in the formation of multinucleated syncytial plaques that increase in size with the progress and demand of the conception [1]. The formation of tri-nucleated hybrid cells and syncytial plaques prevents the fetal cells from direct contact with the maternal blood and the circulatory immune cells [2]. The mono-and bi-nucleated trophoblastic cells release pregnancy-associated glycoproteins (PAGs) by exocytosis, making them available in maternal circulation [3]. The PAG as a biomarker has several disadvantages, such as multiple isoform expression, high glycosylation, longer half-life, and pregnancy stage-specific expression patterns. The discovery of other biomarkers of early pregnancy is thus crucial for developing alternative pregnancy diagnosis methods, determining the prognosis of pregnancy, and early development-related events.

The complex phenomenon of reproduction that happens in the maternal womb is comprising of fertilization, genome activation, early development, hatching, maternal recognition of pregnancy, implantation, development of the body system, adjusting the maternal immune system to prevent conceptus (fetus and placenta) rejection, nutrient and gaseous exchanges, and discharging the excretory metabolic products from the conceptus and delivery. These events have multiple activation/inactivation steps of cell signaling and biochemical pathways that remain largely unknown due to the dynamic nature and non-accessibility of the embryonic tissues from the womb. While both systems continuously communicate to ensure the development of the foreign body, the conceptus, it is expected that some evidence might appear in and disappear from the maternal circulation. Human studies revealed that the conceptus origin circulatory cells [4], proteins [5], and nucleic acids [6] are available in maternal circulation. Except in primates, circulatory cells of the conceptus origin have an ambiguous presence in the other species [7]. The proteins and nucleic acids of conceptus origin have limited life and availability due to their susceptibility to digestion by the circulatory proteases and nucleases. Recently, exosomes have emerged as a field of research, including the discovery of pregnancy biomarkers. An increase in the number of exosomes is observed in *Eclampsia*, a type of abnormal pregnancy in humans [8]. Exosomes contain multifarious bioactive molecules, *viz.*, proteins, nucleic acids, glycoconjugates, and lipids in their cargo that can serve as biomarkers for the diagnosis and prognosis of pregnancy [9]. The cell membrane envelope of exosomes protects the proteins and nucleic acid contents from the circulatory proteases and nucleases digestion [10]. In humans, conceptus-origin exosomes are carried to different parts of the maternal body system via blood circulation for communication and affecting maternal changes [11]. Limited information is available on farm animal species, particularly on buffalo. The discovery of pregnancy-specific exosomes and their content in this species might go a long way as biomarkers and in characterizing events of pregnancy and developmental biology. Among the isolation methods of exosomes [12], precipitation is easy to follow from the complex blood serum matrix, although it might contain some other protein impurities. This study was thus designed to understand whether the precipitated fraction of maternal serum proteins by exosome isolation reagents helps to understand the cycle and early-pregnancy signals by two-dimensional electrophoresis and to identify the differentially expressed protein spots by LC-MS/MS mass spectrometry analysis for their involvement in the different functional pathways and networks.

Materials and Methods

A total of 15 non-pregnant buffaloes aged between 4 to 5 years, with a history of cycling at least once, were used for this experiment. The animals were housed in the Experimental Livestock Unit of the ICAR-National Institute of Animal Nutrition and Physiology (ICAR-NIANP), Bangalore, Karnataka, India, under an intensive management system. The protocols for this experiment were approved by the ICAR-NIANP Institute Animal Ethics Committee (Approval No NIANP/IAEC/08/2012 dated 28/01/2012) and the Department of Biotechnology, Department of Science and Technology, Government of India, New Delhi. All the buffaloes were examined for the presence of ovarian structure and a normal reproductive tract by trans-rectal palpation to qualify them for the experiment. Estrus was monitored morning-evening daily for 48 days by visual observation of symptoms such as restlessness, frequent urination, and allowing to be mounted by other female buffaloes or parading cattle bullocks in the manger or open. Every day, transrectal examinations of the reproductive tracts of all the females were conducted to assess the clinical indication of uterine turgidity and cervico-vaginal discharge before/during the examination. The animals that exhibited all the above clinical indications were considered to be in estrus. Six female buffaloes observed in the cycle in two consecutive times of 19 – 21 days were designated as non-pregnant, and the

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blood serum samples were collected. The buffaloes not seen in estrus by twice-a-day observation and examination were injected with PG and monitored for estrus appearance for the next 5 days. Animals observed in estrus as per the above criteria were inseminated artificially 3 times at 12 h intervals and administered with GnRH (10 µg, Receptal®-2.5mL i/m) at the time of first insemination. The appearance of estrus was tracked similarly from days 15 to 24 post-insemination. The non-responders to the first PG administration and the animals that showed symptoms of estrus after insemination were injected with the second dose of PG on the 11th day of the first PG or cycle, respectively. The appearance of estrus continued to be monitored by observation and clinical examination for the next 5 days. The responder and the non-pregnant cycling animals used for the collection of serum during the estrous cycle were all considered for artificial insemination and GnRH treatment in the next cycle, as described above. Pregnancy of the animals was confirmed by per rectal examination at day 60 post service.

Sample collection, exclusion criteria, and format of analysis

About 10 mL of blood samples were collected in 50 mL centrifuge tubes from the jugular veins of all the selected buffaloes during the cycle (no insemination: days 0, 10, and 15) and pregnancy (retrospective days 15, 30, and 60). The blood samples were allowed to clot at room temperature before being carried to the laboratory and then kept at about 4°C refrigerated temperature for about an hour. Then, the straw-colored serum was separated and harvested by centrifugation at 1500g for 10 min. The collected serum was stored at – 80°C until confirmation of pregnancy status. The samples were not considered for analysis until they were confirmed pregnant by per-rectal examination on day 60 of insemination. The same animals were used to collect non-pregnant and pregnant samples to minimize variability in protein expression across animals. Duplicates of each sample were run in two-dimensional electrophoresis for analysis. The brief analysis done is shown in Figure 1.

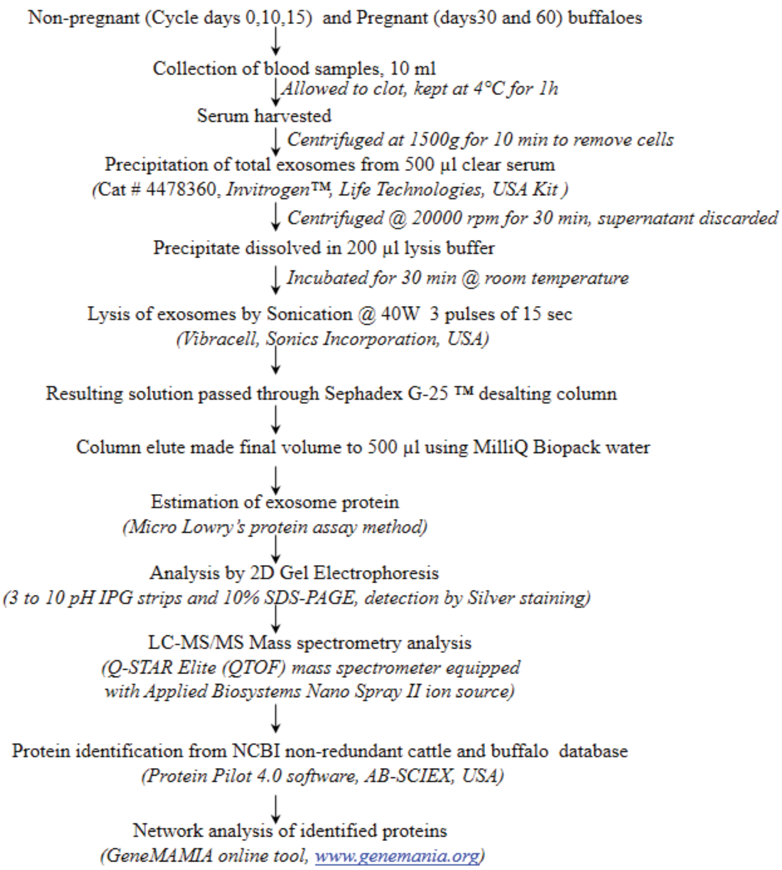

Figure 1: Overview of the protocol followed for serum exosome enriched protein isolation and analysis. Serum exosomes enriched fraction were obtained by the precipitation, exosome protein contents were released by sonication in buffer, and protein quantity estimated and subjected to one (1D) and two-dimensional (2D) electrophoresis analysis. Differentially expressed proteins were identified by the silver staining method. Of these 9 up-regulated proteins in non-pregnant (n=1) and pregnant samples (n=8) were cut out of the gels, and subjected to enzyme digestion, ionization, and LC-MS/MS analysis. The obtained m/z spectra were searched against the cattle and buffalo NCBI non-redundant database. A network was generated based on the functional relationship of the identified proteins.

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Precipitation of serum proteins by exosome isolation reagent

The content of 500 µl serum samples was precipitated using a total exosome isolation reagent (Cat # 4478360, Invitrogen™, Life Technologies, USA). Considering the precipitate will contain the exosomes we followed a lysis protocol by the addition of 200 µl lysis solution (0.5 % Triton-X, 0.1 % SDS, 0.5 % sodium deoxycholate, 10 mM EDTA, 20 mM sodium fluoride) and incubated at room temperature for 30 min and then sonicated at 40 W using three pulses of 15 sec each (Vibracell, Sonics Incorporation, USA). The resulting solution was passed through the Sephadex G-25 column to remove the salt and detergents. The final volume of the solution was made to 500 µl by adding MilliQ Biopack water (MilliQ Direct™, Merck-Millipore, India) before storage in 100 µl aliquots at – 80°C until further use.

Quantification and storage of serum exosome enriched proteins

The total protein in the precipitated sample was quantified by modified Lowry's protein assay [13]. In brief, the desired quantity of 2X Lowry concentrate was prepared by mixing 7.7 % sodium carbonate, 2 % copper sulfate, and 1 % sodium potassium tartarate in a ratio of 13:1:1 just before use. In 3 volumes of this mixture, 1 volume of 10 % sodium lauryl sulfate and 1 volume of 4 % sodium hydroxide (NaOH) were added to prepare the copper reagent. The labeled test tubes were prepared in duplicate, in which the 10 µg, 20 µg, 40 µg, 80 µg, and 120 µg bovine serum albumin (BSA) fraction V and 20 µl samples were taken and made up to 400 µl with double-distilled water (ddH$_2$O). They were allowed to react for 10 min at room temperature with freshly prepared 400 µl copper reagent. To the final reactant 200 µl freshly prepared 0.2 N FC reagent (1:10 dilution of 2N commercial reagent, Cat#062015, SRL, Mumbai, India) was added to develop the blue color in the dark for 30 min and the intensity of color was read at 750 nm wavelength using Microplate reader (Epoch™ Microplate Spectrophotometer, BioTek, USA). The protein concentration in the unknown sample was calculated from the standard curve generated by the optical density of known standard protein concentrations.

Testing of protein quality by one-dimensional electrophoresis

The protein quality in precipitated samples was tested by one-dimensional sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis (PAGE). Gels were prepared following the protocol described in our previous publication [14]. About 80 µg serum precipitated protein was mixed with the 1x reducing sample buffer (0.5M Tris-HCl, pH 6.8, 20% Glycerol, 4% SDS-2g, 2 % β-mercaptoethanol and 0.01% Bromo-phenol blue) in 1.5ml tubes to a final volume of 20 µl and kept in the boiling water bath for 5 min denaturation then transferred immediately to – 20º C for a minute and finally at 4 ºC until loaded in the well for separation under electric field. Along with the samples, standard molecular weight (between ~10 – 220 kDa) protein markers (Cat#10747-012, Bench Mark™, Invitrogen, USA) were also run to determine the molecular weight of the unknown proteins. The proteins were separated at a constant voltage of 40 V until the tracking dye entered the separating gel, and then increased to 70 V until the tracking dye reached the end of the separating gel.

Two-dimensional electrophoresis of the proteins

Isoelectric focusing of proteins:

In the preliminary run, the proteins were found uniformly separated in 7 cm long non-linear gradient strips pH 3 – 10 (Ready strip®; BioRad, USA), thus, we decided to use them for all the sample testing. Before running electrophoresis, about 50 µg protein was mixed with the required volume of rehydration buffer to make a final volume of 125 µl and incubated at room temperature for 1 h. The entire volume was applied as a line along the edge of a channel in the rehydration tray without introducing air bubbles. The coversheet from the IPG strips was peeled off using forceps and gently placed on the specified channel containing the sample, keeping the gel side down and allowing it to soak for 1 h. On top of the strips, 2 ml of mineral oil was overlaid, then the tray was covered with the lid and placed in the IEF apparatus for passive rehydration at 20°C for 12 – 16 h.

A clean, dry IEF focusing tray was prepared by placing paper wicks at both ends of the channel covering the wire electrode, and made wet with 10µl of MilliQ BioPac water. Rehydrated strips from the tray were retrieved, holding the plastic support using forceps, and held vertically on a dry filter paper to drain off the residual mineral oil and unabsorbed proteins. The strips were then transferred onto the focusing tray with the gel side down and covered with 2 to 3 ml of fresh mineral oil. The trapped air bubbles, if any, were removed from beneath the strips. The focusing tray with gel was placed on the IEF cell and focused for 12000 Vh. The protein-focused IPG strips were taken out from the tray, excess buffer and mineral oil were drained off, and the strips were laid on a dry Whatman filter paper. On top of the strip, another wet Whatman filter paper was placed and tapped gently to remove the excess buffer and oil. The IPG strips were then placed on a dry, disposable rehydration tray with the gel side up and used immediately for a second-dimensional SDS-PAGE run. In the tray, each IPG strip was treated with 2.5 ml of equilibration buffer-I (0.05M Tris-HCl, pH 6.8, 6M urea, 2 % SDS, 20 % Glycerol and 50 mg DTT) for 10 min followed by 2.5 ml of equilibration buffer-II (0.05M Tris-HCl, pH 6.8, 6M Urea, 2% SDS, 20% Glycerol,

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62.5mg Iodoacetamide) incubation for 10 min with regular shaking. After completion of equilibration, the buffer was decanted completely, and the strips were washed thoroughly by dipping 2 – 3 times in electrophoresis running buffer taken in a 5 mL centrifuge tube.

Analysis by second dimension electrophoresis

For each IPG strip, one SDS-PAGE gel (1 mm thickness) was prepared, and on top of the stacking gel, a 2D comb was placed to form a well that could accommodate the focused IPG strips. The individual strips prepared the same way were then slid on top of the stacking gel and immobilized by filling the gap with the melted overlay Agarose solution, which was allowed to solidify. The casted gels were then placed into the electrophoresis apparatus and run at 40 V for 45 min, followed by 70 V until the dye front reached the bottom of the gel slab. The proteins in the gel after electrophoresis were detected by mass spectrometry-compatible silver staining methods [15]. The PD Quest 2D image analysis software (BioRad, USA) was used to analyze the silver-stained 2D gel images. From all the duplicate gel images of the cycling and pregnant buffalo samples, six representative gel images were selected for different stages of the cycle and different days of pregnancy. The estrous cycle samples collected on different days were compared with pregnancy samples to understand the unique, up-regulated, and down-regulated protein spots after normalization based on the total density of the gel images. After selecting the representative 6 gel images from each group, the large, small, and faint spots were determined according to the spots present on the gels. The parameters set for analysis were 5 for sensitivity and 7 for size scale to compare the gel images, as the overall intensities of the spots were higher. All the gel images were normalized based on the total density and differentially expressed protein spots (up/down). Differential expressions were determined based on two-fold increases in density ($p<0.05$) along with the other default set parameters of the software. The data generated were stored and used for the selection of spots and mass spectra analysis. A manual, visual approach was also performed, considering it the gold standard [16] for identification of the differentially expressed protein spots.

Mass spectrometry of selected differentially expressed proteins

The protein spots that migrated differently, up-regulated in the cycle and pregnancy, were cut out manually from the silver-stained gels, sliced into smaller pieces, and taken in separate tubes. Gel pieces for each spot were de-stained using freshly prepared 10% sodium thiosulfate and 0.5% Potassium Ferrocyanide solution (about 100 µl) by repeated washing until the gel appeared clear. Following this, the gel pieces were washed several times in a 50 % acetonitrile solution containing 100 mM Ammonium bicarbonate (NH_4HCO_3). Proteins were then reduced using 10 mM Dithiothreitol (DTT) in 100 mM NH_4HCO_3 solution for 45 min at 56°C. The proteins were then alkylated using a solution of 55 mM Iodoacetamide in 100 mM NH_4HCO_3 for 30 min at room temperature in the dark. Finally, in-gel digestion of the spots was carried out using 20 µl (10 ng/µl) of sequencing-grade trypsin in 50 mM NH_4HCO_3 overnight at 37°C. The peptides generated after digestion were extracted in NH_4HCO_3 buffer with 5% formic acid. Samples were vacuum-dried and reconstituted in a buffer with 5% formic acid. The protein digest spectrum was acquired on a Q-STAR Elite (QTOF) mass spectrometer equipped with an Applied Biosystems Nano Spray II ion source. The generated mass (m) to charge (z) ratio peaks were selected for at least 6 amino acids match and queried with NCBI non-redundant protein database of buffalo (*Bubalus bubalis)* and cattle (*Bos taurus*) using the Protein Pilot 4.0 software (AB-SCIEX, USA) with the setting of 10% threshold, precursor and fragment mass tolerances of 0.15 Da, Cysteine Carbamidomethylation as fixed modification and methionine oxidation as variable modification or with the Mascot search engine with similar settings.

Network analysis of the identified proteins

The list of 19 proteins identified by this approach was analyzed through the KEGG mapper online tool (https://www.genome.jp/kegg/mapper/search.html. [17]) using the search mode setting of "hsa", selection of "include aliases (for hsa and other org modes)", and finally clicking on the "exec" to reveal the biochemical and metabolic pathways in which these genes are included. In addition, the gene symbols of the identified protein were further analyzed using the online GeneMANIA tool (www.genemania.org, [18] selecting *Homo sapiens* as an organism. A combined network was generated by selecting the parameters, physical interactions, co-expression, predicted functional relationships, co-localization, genetic interactions, pathways, and shared protein domains.

Statistical analysis

The difference in total protein yield in serum and serum exosome was analyzed statistically by one-way analysis of variance (ANOVA) using SPSS software (Version 16, Chicago, USA). The data were expressed as mean values with standard error. The differences in means at the $p<0.05$ level of significance were obtained by Duncan's post hoc test.

Results

Comparison of protein concentration in neat serum protein and its precipitated fraction in cycling and pregnant buffaloes

The buffalo serum contained, on average, 291.37 mg/mL (n=15; range 251.5 to 319.5 mg/mL) total proteins during the estrous cycle and early pregnancy as per the micro Lowry's

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protein estimation. The precipitated serum protein fraction obtained by the exosome isolation reagent contained about 28.84% (range 25.8 to 32.8) of the total serum proteins. The availability of total protein in serum was lower (p≤0.05) in day 10 (mid-luteal) buffaloes than in day 0 (estrus) and day 15 (late-luteal) samples (Figure 2). However, the day 30 and 60 pregnant samples contained a similar amount of protein as on any day of the cycle. The comparison of precipitated serum protein revealed that day 10 contained significantly lower proteins than in all the other stages of the cycle and early pregnancy (Figure 2).

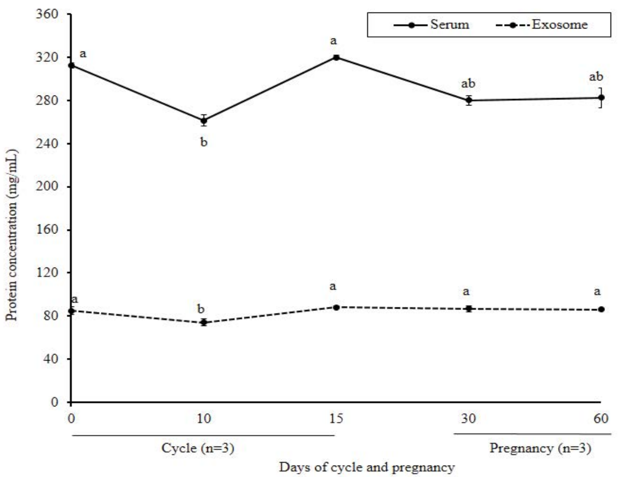

Figure 2: Line diagram showing the changes in protein concentrations in buffalo serum and isolated serum exosome at different days of the cycle and early pregnancy.

The protein concentration (mg/ml) in serum and serum exosome samples during different days of the cycle and pregnancy was estimated by Micro-Lowry's method. Line A: Comparison of serum protein yield. Line B: Comparison of serum exosome protein. The letters "a", and "b" on top of each point indicated significant differences among the groups, whereas "ab" indicated no difference at p<0.05.

Differential expression of protein spots in different days of the estrous cycle and pregnancy samples by two-dimensional electrophoresis analysis

The precipitated serum proteins were separated well in the 7 cm nonlinear 3 to 10 pH gradient IPG strips and the second dimension SDS-PAGE. However, more protein spots were observed near the 90 kDa and 40 kDa regions, and the intensities of protein spots were also higher in that area. Comparison of profiles in different stages of the cycle vs pregnant samples revealed many up and down-regulated (p<0.05) spots (Figure 3). Comparison between day 60 pregnant vs. day 10 cycle samples revealed 17 differential expressions (p<0.05), out of which 12 were up-regulated and 5 were down-regulated (Table 1). The 12 up-regulated spots were the maximum among all other comparisons. Comparison of day-15 vs day-30 and 60 pregnant samples resulted in the up-regulation of 9 spots. An increasing trend of up-regulation (p<0.05) of spots was observed with the increased days of pregnancy from day 30 to day 60 when compared to any of the days (0, 10, and 15) of the cycle. A comparison of day 30 vs day 60 pregnant samples showed 6 up-regulated and 8 down-regulations. Compared to day 0-cycle samples, there were only 2 spots down-regulated in the day 10 and 15-cycle samples. Compared to day 10, the day 15 samples had 7 down-regulated spots and only one up-regulated spot (Table 1). A total of 8 pregnancy-specific unique spots were observed in the day 30 and 60 samples by comparing the day 0, 10, and 15 of the cycle samples. Although some spots were significantly up-regulated (p<0.05), no unique spots could be detected in the day 0 cycle vs. day 10 of the cycle, day 10 cycle vs. day 15 of the cycle, and day 30 pregnant vs. day 60 pregnant comparisons (Table 1). The day 30 pregnant sample had only up-regulation and no down-regulation compared to the day 0 and day 15 of the cycle samples.

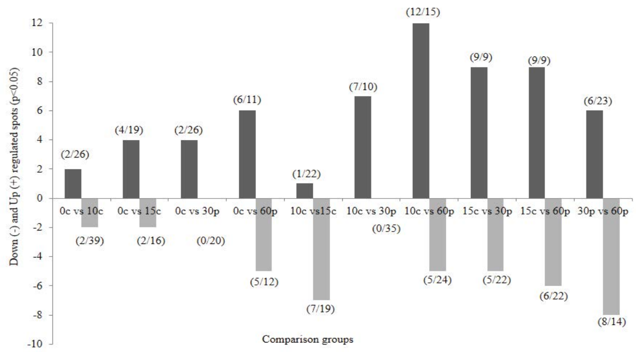

Figure 3: Bar diagram showing the number of differentially expressed protein spots in gels compared to the samples of different days of the cycle and early pregnancy in all possible combinations. The (+) and (-) bars along the X-axis indicate the number of up- and down-regulated protein spots, and the different days of the cycle (c) and early pregnancy (p) used for comparison are shown on the Y-axis. The spot numbers considered for plotting were differentially expressed (p<0.05) and are mentioned in brackets along with the total number of spots under the up and down regulation categories.

Mass spectrometry analysis of the selected differentially expressed protein spots

The LC-MS/MS analysis conducted on 8 pregnancy-specific unique and one cycle-specific up-regulated spots (Figure 4) revealed the identity of 19 different proteins in the combined NCBI nr buffalo and cattle protein databases. Of 19 proteins, 4 were uncharacterized but named based on their presence in cattle and buffalo whole genome assembly and similar sequence characteristics of other species. Five proteins were found to be part of the different signaling pathways (Sl No 1, 2, 5, 6, 10), and the remaining 10 were implicated in many biological functions (Table 2a and

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Table 1: Number of differentially expressed protein spots in different days of cycle (c) and early pregnant (p) buffalo serum exosome

Days of cycle (c) or pregnancy (p)	Up regulation		Down regulation		Unique	Overall differential expression	
	Total	Significant (p<0.05)	Total	Significant (p<0.05)		Total	Significant (p<0.05)
0c vs 10c	26	2	39	2	Nil	65	4
0c vs 15c	19	4	16	2	2	35	6
0c vs 30p	22	4	20	0	3	42	4
0c vs 60p	11	6	12	5	2	23	11
10c vs 15c	22	1	19	7	Nil	41	8
10c vs 30p	10	7	35	0	4	45	7
10c vs 60p	15	12	24	5	4	39	17
15c vs 30p	9	9	22	5	1	31	14
15c vs 60p	9	9	22	6	1	31	15
30p vs 60p	23	6	14	8	Nil	37	14

b). Two proteins, PDK1 (pyruvate dehydrogenase (acetyl transferring) kinase isozyme 1, mitochondrial isoform X1), and SLAM9 (Signaling lymphocytic activation molecule family 9); and MARK1 (Serine threonine protein kinase 1) were identified in two spots. These two proteins and the proteins in spot 4 (Etoposide-induced protein 2.4 homolog and Kallikrein-12) were identified only in the buffalo database. However, the proteins of spots 1, 2, and 3 could only be identified in the cattle database (Table 2a and b). Most of the proteins identified had good confidence scores; however, the gel molecular weight and pI values of many proteins did not match with the theoretical molecular weight and pI calculated based on the coding region amino acid sequences of the identified proteins.

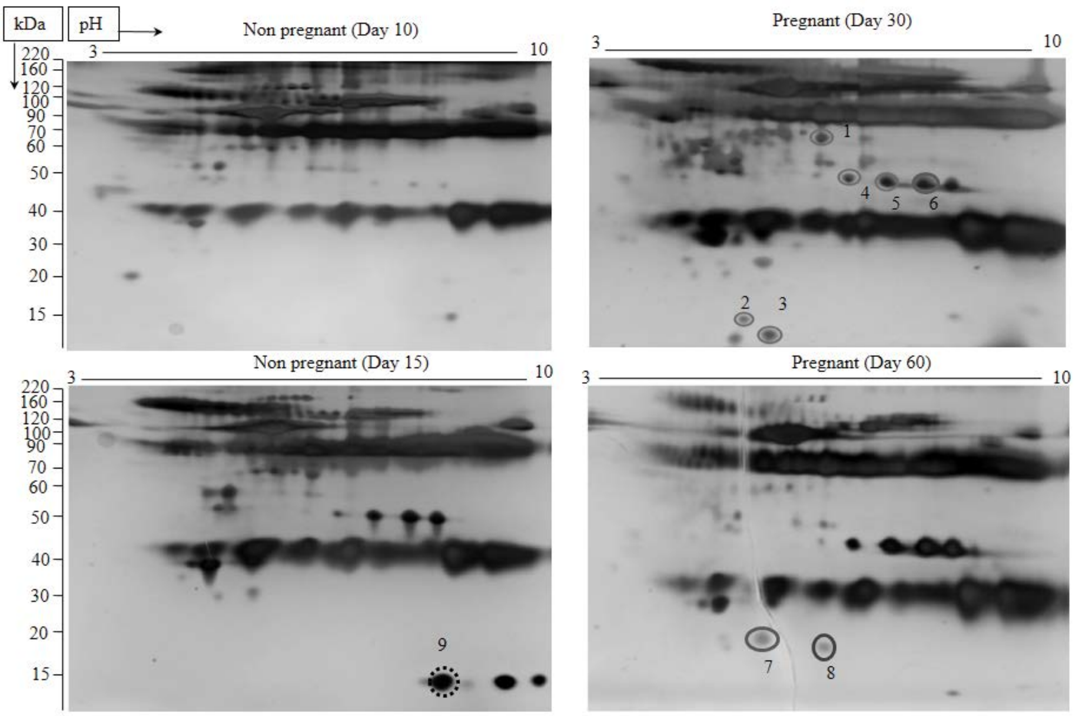

Figure 4: Silver-stained SDS-PAGE gel image showing the positions of the differentially expressed protein spots in early pregnant and cycling buffalo serum exosome samples. The 8 pregnancy-specific spots are circled with grey circles, and one cycle-specific spot is marked with a dotted circle. All 9 spots were cut from the gel, de-stained, and fragmented using enzyme digestion, ionized, and analyzed by LC-MS/MS. The direction of the pH scale of the first dimension isoelectric focusing and the migration patterns of the standard molecular weight proteins, as observed in a similar gel, are shown on the left scale. The days of the cycle and pregnancy of the samples analyzed are mentioned at the top of the images.

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Table 2a: List of the 9 proteins identified from the selected differentially expressed serum exosome protein spots with their NCBI protein accession number, matched peptide, and known/predicted functions as per the available literature.

Sl No	Spot ID	Identity of proteins in *Bos taurus*	Peptides	NCBI Acc. No.	Functions
1	1&8	PREDICTED: REVERSED pyruvate dehydrogenase (acetyl-transferring) kinase isozyme 1, mitochondrial isoform X1 (PDK1)	LFMFSTK	XP_010800201.1	Regulates the catalytic activity of pyruvate decarboxylation oxidation via the mitochondrial pyruvate dehydrogenase complex (Wang et al., 2021)
2	1	PREDICTED: Actin-related protein 2/3 complex subunit 1A (ARPC1A)	WSPLENK	XP_005225118.1	Regulation of actin cytoskeleton, cell proliferation, migration, and invasion (Laurila *et al.,* 2009)
3	2	PREDICTED: Exonuclease mut-7 homolog isoform X3 (EXD3)	LERLSPR	XP_010808893.1	Predicted to be involved in nucleic acid phosphodiester bond hydrolysis. (https://www.ncbi.nlm.nih.gov/gene/549)
4	2	Embryonic stem cell-specific 5-hydroxymethyl cytosine-binding protein isoform X3 (HMCES)	TSCHLPR	XP_005223235.1	A DNA damage recognition protein that preserves genome integrity by facilitating the repair of basic sites (AP sites) in single-stranded DNA (ssDNA) (Mohni et al., 2019).
5	3	PREDICTED: NAD-dependent protein deacetylase sirtuin-3, mitochondrial (SIRT3)	FPRSAVPALR	XP_010799033.1	Mitochondrial de-acetylase (Lombard *et al.,* 2007)
6	3	PREDICTED: Integrator complex subunit 1 isoform X1 (INTS1)	SLLAGLSLPSR	XP_005225307.1	Mediates 3-prime end processing of small nuclear RNAs U1 and U2 (Baillat *et al.,* 2005).
7	3	PREDICTED: lisH domain and HEAT repeat-containing protein KIAA1468 homolog isoform X5 (KIAA1468/*RELCH)*	ADLVREAVIK	XP_005224427.1	Regulates intracellular cholesterol distribution through interactions with RAB11and and oxysterol-binding protein (Sobajima *et al.*, 2018)
8	3	REVERSED: TPA: myeloid/ lymphoid or mixed-lineage leukemia 2-like (MLL2/ KMT2D)	VQKQAEAALR	DAA29966.1	Epigenetic marking- responsible for the generation of H3K4me1, H3K4me2, and H3K4me3 marks, induce gene activation including multiple HOX genes essential for normal development (https://www.genecards.org/cgi-bin/ carddisp.pl?gene=KMT2A)
9	3	PREDICTED: REVERSED Ras-related protein Rab-36 isoform X2 (RAB36)	RSDPTEPRR	XP_010812562.1	Associated with Golgi apparatus, regulates the spatial distribution of late endosomes and lysosomes (Chen *et al.*, 2010)

Table 2b: List of the 10 proteins identified from selected differentially expressed serum exosome protein spots with their NCBI protein accession number, matched peptide, and known/predicted functions as per the available literature.

Sl No	Spot ID	Identity of protein in ***Bubalus bubalis***	Peptides	NCBI Acc No	Functions
10	4	PREDICTED: etoposide-induced protein 24 homolog (EI24)	GIEMHQR	XP_006057733.1	An immediate-early response gene directly regulated by p53, suppresses cell growth and induces cell death (Gu *et al.*, 2000).
11	4	PREDICTED: kallikrein-12 isoform X3 (KLK12)	LGEHSLSK	XP_006080068.1	Subgroup of serine proteases, related to Keratinization and collagen chain trimerization. (https:// www.genecards.org/cgi-bin/carddisp.pl?gene=KLK12)
12	5&9	PREDICTED: Signaling lymphocyte activation molecule (SLAM) family member 9 (SLAMF9) ***Bos taurus***	LATVVPEK	XP_006080977.1	Co-receptors for lymphocyte activation and/or adhesion (Fennelly *et al.,* 2001)
13	5	REVERSED proline-rich protein 12 isoform X1 (PRR12) ***Bubalus bubalis***	TPKRGRR	XP_005195471.1	Predicted to have a role in early central nervous system development, (https://www.omim.org/entry/616633 ?search = PRR12)
14	6 &7	PREDICTED: serine/threonine-protein kinase MARK1 ***Bos taurus***	GLEVLSPR	XP_006080837.1	Phosphorylation of microtubule-associated proteins (Drewes *et al.,* 1997)
15	8	Synaptotagmin-10 (SYT10)	KKFQTR	NP_001179841.1	Predicted in phospholipid binding activity, protein dimerization activity, and syntaxin binding activity, active in exocytic vesicle and plasma membrane. (https:// www.genecards.org/cgi-bin/carddisp.pl?gene=SYT10).

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16	8	Coiled-coil domain-containing 102A (CCDC102A)	KQFQEK	AAI26687.1	Predicted to be part of the myosin complex (https://www.ncbi.nlm.nih.gov/gene/92922)
17	8	PREDICTED: collagen alpha-2 (VI) chain isoform X2 (COL6A2)	GDPGDAGPR, KLIKESR	XP_010799975.1	Muscle tissue formation (Weil *et al.,* 1988)
18	8	Actin-related protein 2/3 complex subunit 1B (ARPC1B) ***Bubalus bubalis***	IYASFIR	NP_001014844.1	Control actin polymerization and cell migration (Laurila *et al.,* 2009).
19	9	PREDICTED: serine/threonine-protein kinase 36 (STK36)	EAALVALR	XP_006048232.1	Act synergistically with GLI2 to activate SSH pathways (Murone *et al.,* 2000)

KEGG mapper classification of the identified proteins and network analysis

In the KEGG mapper analysis, 13 proteins could be classified under different categories, whereas 6 remained unclassified (Figure 5). The maximum number of proteins was enzymes (n=6), of which protein kinases (n=3) were the maximum, followed by cytoskeleton proteins (n=3), chromosome and associated proteins (n=3), and membrane trafficking (n=3). The other categories were peptidases and inhibitors (n=1), glycosaminoglycan binding proteins (n=1), cilium and associated proteins (n=1), exosome (n=1), GTP-binding proteins (n=1), and Spliceosome (n=1). In functional network analysis, two proteins were not connected to defined

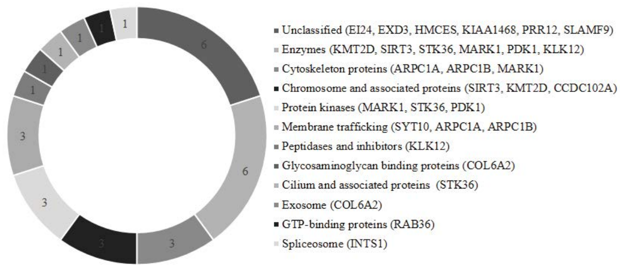

Figure 5: Doughnut diagram showing the classification of identified proteins by KEGG mapper online tools. Out of the total 19 proteins, 6 proteins could not be classified, whereas the remaining 13 proteins could be classified under 11 different categories, of which the enzyme category was the maximum.

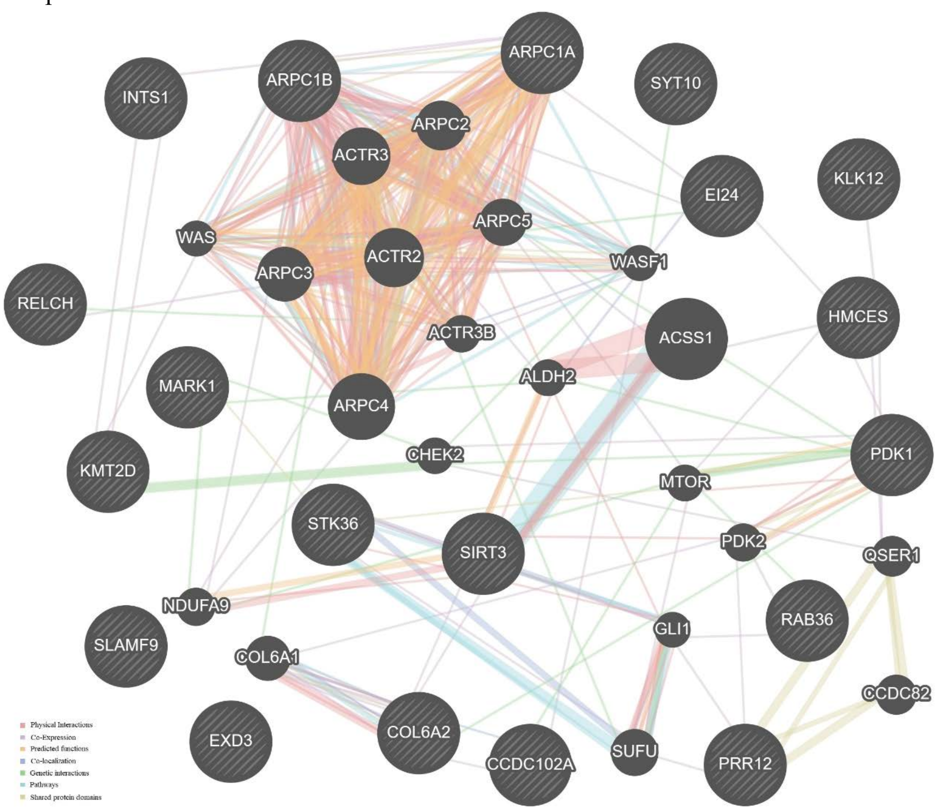

Figure 6: The network generated by the list of the identified 19 genes given as input (indicated with stripes). The final network was the outcome of the interactions of 17 proteins, as two of the proteins were outside the network due to unrelated properties of the rest of the proteins. The lines with different colors signify interactions due to different properties, as indicated by the color palette at the bottom left of the picture.

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functional categories. The remaining 17 identified proteins could form 682 related links with another 19 proteins to form the presented network (Figure 6). They were linked either due to the physical interaction (77.64%), co-expression (8.01%), predicted functional interactions (5.37%), co-localization (3.63 %), genetic interactions (2.87%), pathways (1.88%), and shared protein domains (0.60%).

Discussions

Dynamic molecular and cytological events in the maternal womb during pregnancy have largely remained unknown due to non-accessibility of the maternal and conceptus tissues required to analyze during different days of pregnancy. Maternal circulatory blood contain a treasure of information, which might reflect the cross-talk between the conceptus and the maternal system if explored properly. However major problem is that the early conceptus released bio molecules when gets diluted in the huge quantity of maternal blood that might skip detection even by highly sensitive quantitative assays. This necessitate enrichment of signal before analysis. We thus precipitated the maternal serum proteins using a commercial exosome isolation kit that used precipitation principle. The 2D analysis of these precipitated protein revealed subtle changes in protein profiles of exosome enriched serum protein fractions during cycle and pregnancy in buffaloes. In the 9 unique spots, 19 proteins could be identified by LC MS/MS analysis whose functions were related to conceptus growth and development. These information is invaluable and reports for the first time the availability of conceptus growth and development signal in maternal circulation of buffaloes.

It has been explained that the commercial serum exosome precipitation reagent might co-precipitate other proteins along with exosomes [12]. Keeping the above and the evidence that the recovery of protein in the precipitated fraction was about 28% of the total serum protein (Figure 1) we termed this precipited protein fraction as the exosome-enriched maternal serum protein. The 2D analysis of this exosome-enriched maternal serum proteins of the estrous cycle and pregnancy days revealed signals of the respective stages; thus, the findings are unique. This could be one of the easiest methods of enriching the conceptus signal from the maternal serum proteins that represent a combined maternal and conceptus systems.

The variations of the total serum protein concentration during the estrous cycle and pregnancy observed in our study are reported in cattle [19]. In cattle, the estrus (day 0) serum samples contain a lower total protein concentration than in the other phases of the estrous cycle due to the reduction in serum alpha-1, gamma-1, and gamma-2 globulins [19]. The quantitative differences in the individual constituents of the serum proteins that might have led to lower total serum and precipitated proteins on day 10 of the cycle than on day 0 and day 15 of the cycle could not be enumerated in our study. The protein concentration obtained by the micro-Lowry method in the buffalo serum of our study is more than the reported values [20]. This overestimation might happen due to the pipetting error that occurred while diluting the samples 50 to 60 times to bring the estimation ranges of 0.01 to 1 mg/mL for analysis. A biuret assay that can estimate the serum protein without dilution would have been ideal, as used by others [20]. The use of bovine serum albumin fraction-V (BSA-fraction V) as a standard might also have contributed to the reported overestimation values [21] in the sample. These errors, however, did not affect the equal loading of the protein sample during electrophoresis as reflected by the uniform density of the reference protein, the serum albumin band for all the samples in the gel (Supplementary Figure 1). The 1D electrophoresis analysis could not delineate the differences in serum and serum exosome protein profiles (Supplementary Figure 1) as the separation of proteins was based on the differences in molecular weights. A protein band in the 1D gel may represent different functional proteins, and a single band might represent multiple proteins. In contrast, subtle differences were revealed in two-dimensional gels (Figure 4) that employed protein separation by the isoelectric focusing in one dimension and then by SDS-PAGE in the second dimension. The uniform distribution of protein spots in different 2D gels was the proof, and that ensured a good comparison of differential expression analysis of exosome proteins. Multiple protein spots in 2D gels in a specific molecular weight are the proteins that appeared as a single band in one-dimensional electrophoresis for that particular molecular weight.

Our results indicated that the precipitated serum contained proteins that are key to understanding the transitional events of the cycle and pregnancy at day 15 (Figure 3). The down-regulation signal of proteins at day 15 of the cycle has greater significance as this day is considered the critical for cattle and buffaloes as pregnancy recognition happens in and around this day when the fate of the CL is decided. In ruminants, syncytiotrophoblast produces pregnancy-associated glycoproteins (PAGs) [3] and release them into the maternal circulation by exocytosis. In human syncytiotrophoblasts, the cells of the placenta release exosomes directly into maternal circulation that act as a major signaling mechanism between fetus and mother [22]. Since syncytiotrophoblast formation is common in human and ruminant pregnancy, exosomes of the conceptus origin must also be present in the ruminants same way as in human. Transmission electron microscopy and flow cytometry are the two methods used for the identification of the serum exosomes of pregnant women [23]. Both techniques are used to understanding the increase in the total number of exosomes (not the relative number/mL) in the serum of days 14 to 16 in pregnant mares as compared to the days 12 to 14 in early-pregnant and non-pregnant mares [24]. However, only the number of exosomes present during early pregnancy may not reflect the true function or the cargo they carry [24]. Our investigation on the exosome-enriched serum proteins by 2D electrophoresis was aimed to understand the differentially expressed protein spots and identifying the proteins by LC-MS/MS mass spectrometry analysis. The serum proteins was analyzed for the first time by two-dimensional (2D) electrophoresis and MALDI-TOF MS in Holstein cows between days 21 and 31 of pregnancy that resulted in the identification of nine differentially expressed proteins, including transferrin, albumins, IgG, and gamma globulins [25]. Later Cairoli *et al.* [26] used one-dimensional (1D) and 2D electrophoresis with tandem HPLC-MS to report the most abrupt changes in protein expression at the end of pregnancy and early postpartum. A more advanced technique, 2-DE isobaric tags for relative and absolute quantitation (iTRAQ) and liquid chromatography-tandem mass spectrometry (LC-MS/MS), is used by Li *et al.* [27] in the blood serum of Holstein cows during the periparturient period. Their study revealed a changed expression of 19 of 78 proteins. The few up-regulated proteins one day after delivery were conagglutinin, apolipoprotein A-II, deoxyhemoglobin, and ECM1, and down-regulated proteins were haptoglobin and lipopolysaccharide-binding proteins when compared to day 21 before delivery. Up-regulation of at least 65 protein spots has been reported in major proteins depleted serum proteome analysis during different stages of early pregnancy in buffaloes [28]. This study claimed some of the identified spots as promising pregnancy biomarkers, especially synaptojanin-1, apolipoprotein A-1, apolipoprotein B, keratin 10, and Von Willebrand factors, which have a known role in embryogenesis and early pregnancy maintenance [28]. So far, no report is available on profiling of the exosome-enriched serum proteins in buffalo that revealed differences in protein profiles at days 30 and 60 of pregnancy. Our approach differs from approaches of the depletion of the most abundant proteins, such as serum albumin and immunoglobulins, using an immuno-affinity matrix [28]. The major protein depletion method adopted the immuno-affinity approaches [29]. Using immune depletion approach some proteins are identified in human, rodent, and cattle studies that were part of the regular metabolic pathways, and enzymes of normal cell and body functions. This approach suffers from the inherent disadvantages of first, the removal of other proteins that normally interact with serum albumin and immunoglobulin due to the alternate charge properties. Second, species-specific immuno-affinity matrices due to non availability of human or rodent kits, are used which may not work efficiently for the removal of major proteins. Lastly, many important proteins might be removed due to non-specific interactions with the column matrix and the albumin and immunoglobulin. This way multiple pregnancy-specific signals might be partially or completely removed depending on the type of interaction. Therefore, the information generated in such studies needs to be interpreted carefully. A sensitive quantitative proteomic analysis available at present [30] might reveal a better picture as it provides the opportunity for low-abundant protein detection. We argue that any technique are useful if it produces biologically important data. In the current experiment we could discover significant differential expression of proteins depending on the changes in estrus cycle and pregnancy days. In addition, at least 4 unique and 4 significantly up-regulated pregnancy-specific protein spots by comparing cycling animals' 2D protein profiles (Figure 3). The sensitive silver staining method has been helpful to detect the low-abundant proteins in the gel. Since we precipitated the proteins, we did not add protease inhibitors to the sample, as the isoelectric focusing gets affected by the protease inhibitors [31]. However, to minimize the protease exposure and action, the original serum samples were processed as fast as possible, and all the processing steps were carried out at a cool temperature.

The identified proteins with this approach have known functions in many biochemical pathways, such as HIF-1 (*PDK1*), and p53 signaling (etoposide-induced protein 2.4 homologs [32]; spliceosome (*INTS1*); Fc gamma R mediated phagocytosis, bacterial invasion of epithelial cells (*ARPC1A*); central carbon metabolism in cancer (*PDK1* and *SIRT3*). In addition, we also found proteins to have a role in neural development (*CCDC102A*, [33] and function (*SYT10*) [34] and muscle tissue formation (*COL6A2*) [35]. Signaling lymphocyte activation molecule family member 9 (*SLAMF-9*) acts as a co-receptor for lymphocyte activation and/or adhesion [36]. There have been GTP-binding proteins (*RAB36*) [37], a subgroup of serine proteases (Kallikerine 12) [38], and a protein involved in phosphorylation of microtubule-associated proteins (*MARK1*) implicated in the regulation of cell shape and polarity during differentiation, chromosome partitioning at mitosis, and intracellular transport [39]. Also, a protein called serine/threonine kinase 36 (*STK36*) was found that acts synergistically with another protein called Glioma-associated oncogene 2 (*GLI2*) to activate Sonic Hedgehog (*SHH*) pathways [40]. The SHH pathway activation is significant for embryonic development. Interestingly, the SHH protein has been detected in non-pregnant buffalo samples at day 15 of the cycle (Spot #9, Fig 4), which is considered critical for luteolytic and anti-luteolytic signaling in the uterus. The identified proline-rich 12 (*PRR12*) encodes a 211-kDa nuclear protein with suspected DNA-binding activity that is highly expressed in mouse and human brains, particularly in early development [41, 42]. The coding sequence of *PRR12* is well-conserved

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among vertebrates [43]. Functional network analysis of all 19 proteins (Figure 6) revealed Actin-related protein 2/3 complex, subunit 1A (*ARPC1A*), and subunit 1B (*ARPC1B*) proteins which are important regulatory subunits of the seven-member Arp2/3 protein complex (*ARP2/3*) [44] and key regulators of multiple pathways (Figure 5). The *ARPC1A* and *ARPC1B* are members of the *SOP2* (suppressor of profilin-2) family proteins that have WD (tryptophan and aspartate) repeat-containing domains. They regulate actin polymerization and, together with an activating nucleation-promoting factor (NPF), mediate the formation of branched actin networks. Silencing of *ARPC1A* by small interfering RNA reduced cell proliferation, migration, and invasion in a pancreatic cancer cell line with a high level of amplification, indicating that they are essential for cell viability. In contrast, silencing of *ARPC1B* decreased cell migration but did not affect cell proliferation and invasion [45]. An inherent role for *ARPC1B* is evidenced in the regulation of mitosis from the fact that its depletion inhibits Aurora-A kinase activation at the centrosome and impairs the ability of mammalian cells to enter mitosis [46]. Aurora-A kinases play an important role in the regulation of spindle formation and cell mitosis. Other than regulation of actin dynamics, these two proteins are involved in phagocytic cup formation, signaling by Rho GTPases, EPHB (Ephrin B receptor)-mediated forward signaling, membrane progesterone receptor (MPR) pathway, Actin pathway, *CDC42/RAC* (cell division cycle protein-42/ Ras-related GTPase) pathway, and Salmonella pathway. The signaling by Rho GTPases is important as they control almost all fundamental cellular processes in eukaryotes, including morphogenesis, polarity, movement, cell division, gene expression, and cytoskeleton reorganization [47]. The *EPHB*-mediated forward signaling is important for craniofacial morphogenesis, controlling cell proliferation across Ephrin boundaries [48]. The MPR is a non-classical membrane-associated, high-affinity progesterone receptor-mediated rapid activation of the intracellular signaling pathway, which induces cellular responses that are often non-genomic but may also result in alterations in gene transcription [49, 50] through the activation of G-proteins [51]. Taking together all the facts of the identified protein functions, it is apparent that they are essential for the development of the conceptus and might better be considered as the conceptus signals in maternal circulation, not the maternal changes due to the presence of the conceptus. It needs to be confirmed if such signals were present in the exosome cargo or in the co-precipitated other proteins. Moreover, the maternal serum protein mixture represents the maternal and conceptus systems.

In conclusion, we reported here the differences in the protein profiles of the exosome-enriched maternal serum between the days of the cycle and early pregnancy in buffalo by simple two-dimensional electrophoresis analysis and identified 19 proteins from the 9 differentially expressed protein spots. These proteins have known roles in cytoskeleton organization, microtubule dynamics, gene transcription, morphogenesis, brain development, cell cycle progression, mitosis, and cell migration. Such functions are relevant only for the growth and development of the conceptus not the signals of maternal changes induced by the conceptus.

List of Abbreviations

MRP = Maternal Recognition of Pregnancy; IFNτ = Interferon tau; PAGs = Pregnancy-associated Glycoproteins; PG = Prostaglandin; GnRH = Gonadotropin-Releasing Hormone; SDS = Sodium dodecyl sulphate; PAGE = Polyacrylamide gel electrophoresis; EDTA = Ethylene di-amine tetra-acetate; NaOH = Sodium Hydroxide; BSA = Bovine Serum Albumin; FC reagent = Folin Ciocalteu reagent; IPG = Immobilized pH gradient gel; IEF = Isoelectric focusing; 1D = One-dimensional; 2D = Two-dimensional; DTT = Dithiothreitol; QTOF = Quadrupole Time-of-Flight; MS = Mass spectrometry; NCBI = National Center for Biotechnology Information; KEGG = Kyoto Encyclopedia of Genes and Genomes; ANOVA = Analysis of Variance; SPSS = Statistical Package for the Social Sciences; LC-MS/MS = Liquid Chromatography with tandem mass Spectrometry; BSA = Bovine Serum Albumin; MALDI-TOF = Matrix-Assisted Laser Desorption/Ionization Time-of-Flight; IgG = Immunoglubulin-G; HPLC = High-performance Liquid Chromatography; iTRAQ = Isobaric Tags for Relative and Absolute Quantification; *HIF-1* = Hypoxia-inducible factor 1 (HIF-1); *PDK1* = Pyruvate Dehydrogenase Kinase 1 *INTS1* = Integrator Complex Subunit 1; *ARPC1A* = Actin-Related Protein 2/3 Complex, Subunit 1A; *SIRT3* = Sirtuin 3; *CCDC102A* = Coiled-Coil Domain-Containing Protein 10 2A; *SYT10* = Synaptotagmin 10; *PRR12* = Proline Rich 12; *ARPC1A* = Actin-Related Protein 2/3 Complex Subunit 1A; *ARPC1B* = Actin-Related Protein 2/3 Complex Subunit 1B; *ARP2/3* = Actin-related protein 2/3; *SHH* = Sonic Hedgehog; *GLI2*= Glioma-associated oncogene 2; *STK36* = Serine/ threonine kinase 36; *MARK1* = Microtubule-associated proteins 1; *RAB36* = Ras-Related Protein -36; *SLAMF-9* = Signaling Lymphocyte Activation Molecule Family Member 9; *COL6A2* = Collagen Type VI Alpha 2 Chain; *NPF* = Nucleation-Promoting Factor; *SOP2* = Suppressor of Profilin-2; *EPHB* = Ephrin B; *MPR* = Membrane Progesterone Receptor; *CDC42/RAC* = P21 Protein (Cdc42/Rac)-Activated Kinase 2 (PAK2)

Author Contributions

Conceptualization, J.G. and S.K.D.; methodology, J.G and V.R.J.; formal analysis, VRJ, JG, investigation, V.R.J. and J.G.; resource, J.G.; Data curation, J.G., S.K.D.; investigation, V.R.J., JG; Methodology, J.G., V.R.J.; Validation, V.R.J., J.G., S.K.D.; Formal Analysis, V.R.J., JG, SKD; Writing

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Available from the corresponding author.

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