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Porcine salivary proteome analysis identifies potential early pregnancy-specific protein biomarkers

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ABSTRACT

Early diagnosis of pregnancy is of utmost importance to optimize profit in pig husbandry. Identifying candidate protein biomarkers for early diagnosis of pregnancy in a non-invasive sample such as saliva may produce a colossal lead to accomplish the purpose. Therefore, in this study, comparative salivary proteome profile of day 12 of gestation, representing elongation of blastocysts stage and non-pregnant sows was explored by label-free quantitation (LFQ) based mass spectrometry approach to identify early pregnancy biomarkers. A total of 115 proteins were identified as differentially expressed proteins (DEPs) with significant difference between non-pregnant and early pregnancy groups. Among the DEPs, majority of the proteins (82 out of 115 DEPs) were found to be down-regulated in early pregnancy group (fold change >2) compared to non-pregnant control. Functional classification and pathway analysis of the DEPs revealed involvement of most of the proteins in integrin signalling pathways, blood coagulation, carbohydrate metabolism, oxidative stress response and regulation of protein folding. Few DEPs with higher fold change during early pregnancy such as thioredoxin, heat shock 70 kDa protein 1A, alpha 1-S haptoglobin, and glutathione S-transferase pi 1 may have potential as biomarkers for early pregnancy diagnosis in pigs based on their recognized role in different pregnancy related activities. Overall, our results provide a set of salivary proteins which can be used as potential biomarkers for early pregnancy diagnosis after large scale validation.

Keywords: Biomarkers, Differentially expressed proteins, Early pregnancy, Pig, Saliva

Ensuring the pregnancy status at the early stage of gestation has become the top priority for profitable pig production. Conventional methods to accomplish the purpose includes the detection of heat after 18 to 24 days of natural service or artificial insemination (AI) and ultrasound methods such as Doppler, A-mode ultrasound, B-mode ultrasound or real time ultrasonography (RTU). Whereas, the heat detection methods are time consuming and sometimes misleading, the ultrasound methods can produce reliable results only when performed after 24 to 30 days post-service and require an expertise operator. Pregnancy diagnosis based on the estimation of plasma hormones such as progesterone, estrone sulfate, and prostaglandin-F2 as well as monitoring of early pregnancy factor (EPF) activity has also been reported in pigs. However, the lack of statistical difference in the hormone level between pregnant and non-pregnant sows and the non-specific presence of EPF in individuals with a variety of tumors have rendered these methods to be ineffective in the diagnosis of pregnancy in the early stages of gestation.

Successful conception results in the synthesis of a variety of biomolecules including proteins and steroid hormones, many of which are of feto-placental origin (Østrup *et al*. 2010, Bazer 2013, Shen *et al*. 2014). Differential expression of several such proteins has been reported in the endometrium, serum and urine during the early stages of pregnancy in different livestock species including pigs (Chae *et al*. 2011, Zhao *et al*. 2015, De *et al*. 2019). Whole saliva is a mixture of secretions from major and minor salivary glands along with a number of constituents of non-salivary origin. Saliva is composed of hundreds of proteins and peptides, many of which originate from blood due to extravasations and the presence of blood proteins in saliva has been reported in human as well as in pigs (Gutierrez *et al*. 2011, Zhang *et al*. 2013). Therefore, salivary proteins have the potential to reflect any patho-physiological changes of the host. Moreover, saliva as a diagnostic fluid, an alternative to serum, in pigs has several advantages, including non-invasive and stress-free collection of large number of samples and collection from remote areas by unskilled personnel (Prickett and Zimmerman 2010, Ramirez *et al*. 2012).

Earlier studies revealed that by analyzing some potential protein biomarkers, pregnancy can be diagnosed as early as

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12 days post-conception (De *et al*. 2019). It is speculated that the alteration in the serum proteome around 12 days post-conception may also bring changes in the salivary proteome. Hence, in the present study, a comprehensive identification and quantification of the porcine salivary proteome during an early stage of pregnancy was carried out by label-free quantitative proteomics approach to identify potential protein biomarkers.

MATERIALS AND METHODS

Animals and saliva collection: In this study, three clinically healthy Large White Yorkshire (LWY) females were selected from the instructional livestock farm complex of the College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Aizawl, Mizoram, India. The sows in estrus were bred naturally twice, at 24 h interval, and saliva samples were collected using Salivette tubes (Sarstedt) with a sponge as described by Gutiérrez *et al*. (2009), on day 12 post-service considering that the 12th day represents an important early gestational event in sow pregnancy, i.e. elongation of blastocysts. All the saliva samples were mixed with protease inhibitor cocktail (Sigma Aldrich, MO, USA) and stored at −80°C until further analysis. The first day of mating was considered day 0 of pregnancy. All the sows, used in this study, were farrowed after the normal length of gestation with average litter size. Saliva samples were also collected from three non-pregnant LWY females which served as controls. All the animal experiments were carried out after necessary approval from the Institute Animals Ethics Committee (IAEC) of the College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Aizawl, Mizoram, India.

Preparation of protein samples and mass spectrometry analysis: At first, desalting of all the saliva samples was carried out using Vivaspin ultrafiltration spin columns (Sartorius, Germany) as per the manufacturer's instruction. Total proteins from all the desalted saliva samples were separated by acetone precipitation method. Briefly, four volumes of ice-cold acetone were added to one volume of saliva sample. The mixture was vortexed well and incubated for 1 h at -20°C. The tubes were then centrifuged at 15000 × *g* for 20 min at 4°C. The supernatant was removed and the pellet was washed twice with ice-cold acetone (80%) and then centrifugation at 15000 × *g* for 5 min. The final protein pellet was air dried and dissolved in guanidine hydrochloride (GuHCl) buffer (4 M GuHCl, 0.1 M Tris-HCl, *p*H 8.5) for mass spectrometry. Protein concentrations were determined by 2-D Quant protein assay kit as per the manufacturer's instruction (GE Healthcare, NJ, USA). Protein samples were subjected to liquid chromatography coupled to mass spectrometry analysis for their identification and label-free quantitation (LFQ) for comparing the levels of identified proteins between pregnant and non-pregnant groups at Valerian Chem Private Limited, New Delhi, India following the procedure mentioned in our previous study (De *et al*. 2019). Both peptide spectrum match and protein false discovery rate (FDR) were set to 0.01 FDR. Protein identification was carried out based on the detection of at least one peptide with an FDR of less than 1.0%. Relative quantification of the proteins identified in both groups was performed using the Minora feature detector node of Proteome Discoverer 2.2 with default settings and considering only high PSM (peptide spectrum matches) confidence. Protein abundances were calculated on the basis of LFQ intensity. For comparison, the protein abundance in the non-pregnant group was set as a reference and the protein abundance in the day 12 gestation group was aligned. The Z-score normalization of the log2 transformed LFQ values was performed to apply student's t-test for the determination of the significant difference between non-pregnant and day 12 gestation group protein abundances.

Bioinformatics analyses: Using R programming software, protein expression patterns among different groups were presented in a heatmap based on hierarchical clustering by measuring Euclidean distance coupled with the complete linkage method. Functional classification of all the significantly differentially expressed proteins (DEPs) was performed using Protein Analysis Through Evolutionary Relationships (PANTHER) software based on Gene Ontology (GO) against *Sus scrofa* database. Proteins were classified based on molecular function, biological process, cellular component, protein class, and pathway. To understand the possible interactions between the DEPs and visualize the protein-protein interaction (PPI) network, Search Tool for the Retrieval of Interacting Genes (STRING) software was used.

RESULTS AND DISCUSSION

Identification of differentially expressed proteins: Salivary proteins of non-pregnant and pregnant sows (25 µg each) were subjected to mass spectrometric analysis and a total of 274 protein groups were identified commonly in non-pregnant and pregnant sows, considered as DEPs, with high confidence using 1% protein and peptide FDR cut-off. Among 274 DEPs, only 115 DEPs were found to be significantly different (P<0.05) between non-pregnant and pregnant sows (Table 1; Supplementary table 1). Further, comparative analysis of the protein groups of non-pregnant and day 12 of pregnancy revealed 26 and 4 protein groups, respectively, detected exclusively in all three replicates of one experimental group and not in the other (Table 1). Comparison of saliva proteome profiles of non-pregnant and pregnant sows was performed by subjecting the 115 DEPs to Hierarchical Clustering and the resulting clustergram is shown in Fig. 1. Scrutiny of the clustergram indicated distinguishable protein profiles among non-pregnant and early pregnancy groups. Out of 115 DEPs, 13 were found up-regulated and 82 down-regulated, having fold change of more than 2, in the early pregnancy group compared to the non-pregnant control (Supplementary Table 1). Out of 13 up-regulated proteins, some of the proteins showed higher fold change

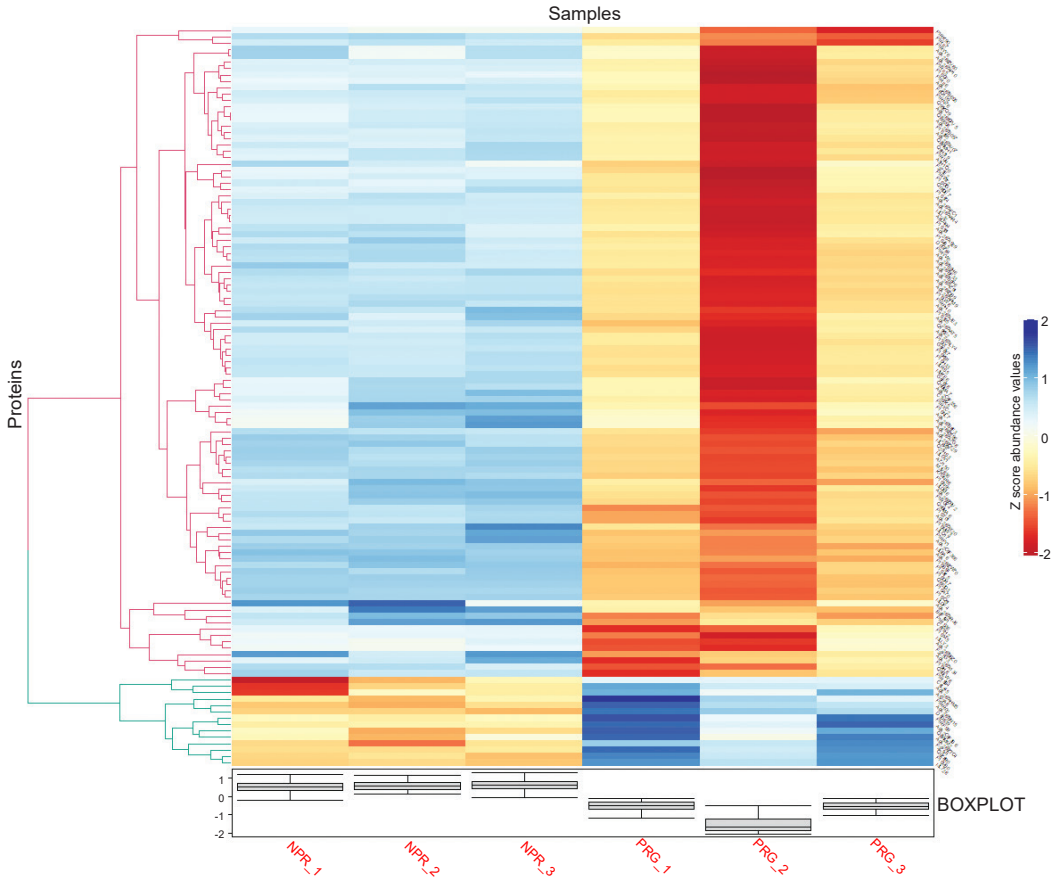

Fig. 1. Clustergram of 115 significantly differentially expressed proteins among the non-pregnant and early pregnant groups. Heatmap was plotted based on hierarchical clustering by measuring Euclidean distance coupled with complete linkage method and represented by blue (high abundance) and red (low abundance) colours as indicated in the colour scale bar.

(>5 fold) in the early pregnancy group compared to non-pregnant control, including cystatin, capping actin protein gelsolin like, thioredoxin, beta-microseminoprotein, etc (Table 2). Similarly, among 82 down-regulated proteins, some of the proteins like pheromaxein A subunit, heat shock 70 kDa protein 1A (HSPA1A), alpha-crystallin B chain (CRYAB), 78 kDa glucose-regulated protein, glutathione S-transferase pi 1, alpha 1-S haptoglobin, complement C3, BPI fold-containing family A member 1, profilin, alpha-1-antichymotrypsin 2, etc. showed more than 5 fold down-regulation in early pregnancy group (Table 3). Analysis of the identified up and down-regulated proteins revealed some important proteins that play a significant role in different pregnancy-related events.

Among the identified DEPs, several proteins including complement C3, alpha-1-antichymotrypsin 2, ceruloplasmin, alpha-1-antitrypsin, alpha-2-macroglobulin, etc. were also reported to be differentially expressed in endometrium and serum during early pregnancy in pigs (Jalali *et al*. 2015, 2016; De *et al*. 2019), which supports our speculation of alteration in the proteome in other tissues due to early pregnancy events also get reflected in saliva. Further, few DEPs identified in our study such as protein S100, fructose-bisphosphatealdolase and complement C3 were also reported to be differentially expressed in human saliva during early and mid pregnancy (Dey *et al*. 2020). However, some of the DEPs found in our study have not been reported previously either due to the difference in biological samples analysed or the proteomic approach adopted.

Up and down-regulation of some proteins with high fold change (>5) in the early pregnancy group indicated their probable involvement in early pregnancy events. For example, thioredoxin, a redox-active defensive protein,

Table 1. Summary of proteins identified and significantly differentially expressed in pig saliva during early pregnancy

Group	Stage of pregnancy	Total proteins identified	Unique A[a]	Unique B[b]	Common[c]	Up-regulated[d]	Down-regulated[e]
1	Non-Pregnant (control)	141	-	26	-	-	-
2	Pregnant (Day 12)	119	4	-	115	13	82

[a]Proteins unique to the early pregnancy stage. [b]Proteins unique to the non-pregnancy stage. [c]The common proteins identified in both the non-pregnant and early pregnancy conditions. [d]The up-regulated proteins in early pregnancy stage (>2 fold). [e]The down-regulated proteins in early pregnancy stage (>2 fold).

Table 2. List of proteins up-regulated in early pregnancy group (Day 12)

Protein ID	Name of the protein (Uniprot/ NCBI)	Gene name (Uniprot/ NCBI)	Fold change (>5)
A0A287AP10	Uncharacterized protein	LOC100125542	6.529
Q0Z8R0	Cystatin	CST3	7.052
F1SVB0	Capping actin protein, gelsolin like	CAPG	9.423
F2Z5J1	26S protease regulatory subunit 4	PSMC1	9.925
A5J2A8	Thioredoxin	TRX	16.097
I3L728	Immunoglobulin kappa variable region	IgV_L_kappa	19.049
A0A2C9F3B6	Beta-microseminoprotein	MSMB	21.258
F1RR02	Glial fibrillary acidic protein	GFAP	100
C6L245	Putative trypsinogen	try	100

Table 3. List of proteins down-regulated in early pregnancy group (Day 12)

Protein ID	Name of the protein (Uniprot/ NCBI)	Gene name (Uniprot/ NCBI)	Fold change (>5)
Q863D3	Pheromaxein A subunit	PHEROA	0.01
F1SQ51	Basic proline-rich protein precursor	TP23	0.01
F1RII5	GLOBIN domain-containing protein	LOC100515788	0.048
P34930	Heat shock 70 kDa protein 1A	HSPA1A	0.066
A0A287ALY4	Alpha-crystallin B chain	CRYAB	0.066
K7GNR8	Hemoglobin subunit theta 1	HBQ1	0.077
F1RGX4	Hemoglobin subunit alpha	LOC100737768	0.087
F1RII7	Hemoglobin subunit beta	HBB	0.089
A7J150	Long palate lung and nasal epithelium protein 2	BPIFB2	0.089
A8U4R4	Transketolase	tkt	0.09
I3LGY1	BPI fold-containing family B member 2 precursor	BPIFB2	0.091
F1RS36	78 kDa glucose-regulated protein	HSPA5	0.095
M3V836	Glutathione S-transferase pi 1	GSTP1	0.109
A0A287AZ91	Glutathione S-transferase P-like isoform X3	LOC100739508	0.109
A0A287AV07	Acetyl-CoA acetyltransferase 2	ACAT2	0.111
I3LDF4	Adenosine deaminase	ADA	0.112
F1RYY6	Transaldolase	TALDO1	0.112
A0A287AFC1	L-lactate dehydrogenase	LDHA	0.115
B0F9U1	Alpha 1 S haptoglobin	HP	0.117
A0A287ARJ8	SERPIN domain-containing protein	LOC100156325	0.116
A0A286ZW70	Peptidyl-prolylcis-trans isomerase	PPIB	0.118
F1SDR7	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein beta	YWHAB	0.12
F1RYW2	Calmodulin like 5	CALML5	0.122
F1S8Y5	Phosphoglyceratemutase	PGAM1	0.123
I3L8W7	Zinc finger protein 180	ZNF180	0.124
I3LRS5	Aldehyde dehydrogenase 1 family member A1	ALDH1A1	0.127
I3LLY3	Actinin alpha 1	ACTN1	0.128
F1SBS4	Complement C3	C3	0.128
F1S505	BPI fold-containing family A member 1	BPIFA1	0.132
A0A287AX20	Cholinesterase	BCHE	0.134
F1RPH0	Phosphoglycerate kinase	PGK1	0.137
A0A287B8F3	Fructose-bisphosphatealdolase	ALDOA	0.14
Q27HS3	Vascular smooth muscle alpha-actin	ACTA2	0.148
A0A286ZRV2	Triosephosphateisomerase	TPI1	0.151
I3LDM6	Keratin 3	KRT3	0.153
F1S501	Vomeromodulin-like isoform X1	LOC102162701	0.164
A0A287AJE3	SERPIN domain-containing protein	LOC106504547	0.166
F1RFY1	Profilin	PFN1	0.17
F1RN76	CD5 antigen-like precursor	CD5L	0.178

(Table 3 *Contd*..)

Table 3. *(Concluded..)*

Protein ID	Name of the protein (Uniprot/ NCBI)	Gene name (Uniprot/ NCBI)	Fold change (>5)
Q9GMA6	Alpha-1-antichymotrypsin 2	SERPINA3-2	0.18
Q19KE2	Fatty acid binding protein 5	FABP5	0.184
F1S395	NAD(P)H dehydrogenase quinone 1	NQO1	0.183
F1SKB1	Ceruloplasmin isoform X1	CP	0.183
I3VKE6	Ceruloplasmin		0.183
A0A287AAT5	SERPIN domain-containing protein	LOC110261637	0.19
F1RWT2	Plastin 3	PLS3	0.193
F1SMW3	Serpin family B member 5	SERPINB5	0.197
F1ST01	Selenium binding protein 1	SELENBP1	0.198

plays a crucial role in detoxification of reactive oxygen species (ROS). Higher expression of thioredoxin was also reported in human decidua and trophoblasts to protect the fertilized egg and placental trophoblasts from the cytotoxic effects of ROS (Di Trapani *et al*. 1998). Hence, higher expression of this protein observed in our study suggesting its possible role in protection of developing pig embryos from the ROS. Down-regulation of few heat shock proteins (HSPs) such as HSPA1A, CRYAB, HSPA5 and HSPB1 was observed in the early pregnancy group in our study. This may be due to the fact that the HSPs can induce an inflammatory response, can serve as an antigenic target for immune system and also can lead to auto-immune mediated reproductive failure. Higher down-regulation of glutathione S-transferases (GSTs) was also observed in early pregnancy group. GSTs are mainly involved in biotransformation and detoxification of xenobiotics and differential regulation of this detoxification protein was also reported in pigs during pregnancy (Chae *et al*. 2012). Further, higher levels of glutathione S-transferase pi 1 was found to be associated with preeclampsia and hemolysis, elevated liver enzymes, low platelets syndrome during pregnancy in human (Knapen *et al*. 1999). The down-regulation was also observed in our study for the alpha 1-S haptoglobin which is an acute phase protein. As per previous report, up-regulation of haptoglobin was observed in pregnant decidua in human might be to protect the fetus from maternal immune-rejection (Berkova *et al*. 2001). However, Gleichmann *et al*. (1973) reported the decrease in serum concentration of haptoglobin due to the effect of estrogen in the oral contraceptives. Hence, the increased estrogen concentration around day 12 of pregnancy in pigs has probably resulted in the down-regulation of alpha 1-S haptoglobin observed in our study.

Functional classification and network analysis of DEPs: All the DEPs (115) were functionally classified based on molecular function, biological process, cellular component, protein class and pathway. Classification based on molecular function revealed the involvement of most of the proteins in catalytic activity (42.4%) followed by binding (35.8%), and other molecular functions. On the basis of biological process, proteins were classified into those involved in cellular process (28.7%), metabolic process (22.8%), and so on. Classification of DEPs on the basis of cellular component shown that majority of the proteins is from cell part (18.5%), cell (18.5%) followed by extracellular region part (14.6%). Further, classification based on protein class revealed that most of the proteins belong to two major protein classes including metabolite inter-conversion enzyme (25.4%) and protein-binding activity modulator (22.2%). Pathway analysis showed the involvement of maximum number of DEPs in blood coagulation (6.3%), glycolysis (6.3%), integrin signalling pathway (6.3%), and other biological pathways. To understand the possible interactions between the DEPs, STRING software was used to build a regulatory network (Fig. 2). The generated network presented proteins as nodes that are linked through edges. Further, k-mean clustering of the protein network revealed 5 distinguished clusters of proteins involved in integrin signalling pathways, blood coagulation, carbohydrate metabolism, oxidative stress response and regulation of protein folding (Fig. 2).

The integrin signalling pathway, commonly obtained in functional classification and network analysis, plays a vital role in the porcine endometrium as proteins of this pathway such as actins and its binding proteins reported to be localized in the endometrium and regulate endometrial cytoskeleton during early pregnancy (Jalali *et al*. 2018). This might be a crucial event during rapid elongation of blastocysts around day 12 of early pregnancy. Regulation of blood coagulation events during early stage of gestation is also key to establish normal pregnancy. The blood coagulation system shifts from anti-coagulant to pro-coagulant state during early pregnancy period to satisfy the hemostatic challenges of placentation (Li and Huang 2009). This may be the reason for the down regulation of serpins in saliva during early pregnancy stage in our study. The regulation of oxidative stress response is also very crucial during the early stage of pregnancy to protect the embryo from oxidative stress and the differential regulation of the antioxidants play vital role in decidualization (Xu *et al*. 2014). Therefore, the significant up-regulation of antioxidants such as thioredoxin and superoxide dismutase observed in our study is aptly justified.

Several proteins identified in our study were proposed as potential biomarkers for early pregnancy diagnosis in different species. Among the proteins identified and found differentially regulated in our study, serpins in cattle urine (Rawat *et al*. 2016), serum albumin precursor and transferrin in cattle serum (Jin *et al*. 2005), fructose-

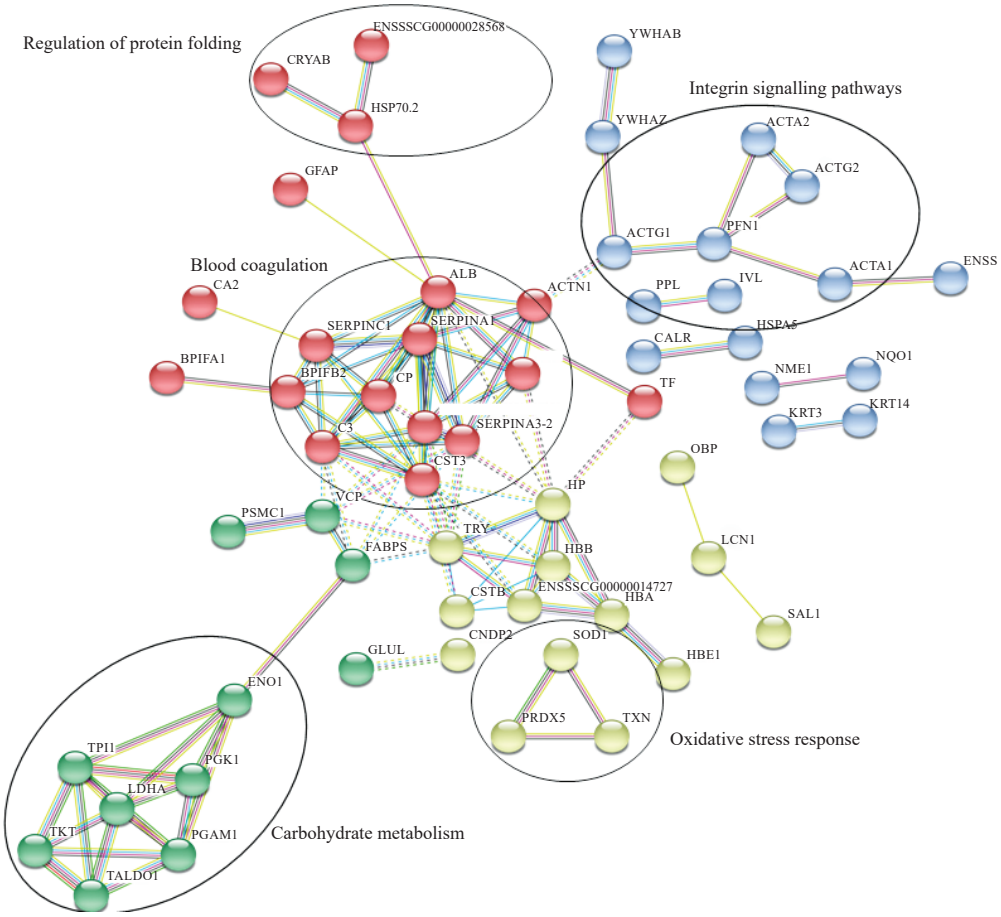

Fig. 2. Network analysis of differentially expressed proteins depicting the possible protein-protein interactions.

bis-phosphate aldolase A and complement C3 in saliva of human (Dey *et al*. 2020) and ceruloplasnin in urine of giant panda (Willis *et al*. 2011) were suggested previously as potential biomarkers of early pregnancy in respective species.

This study presents the first report on global salivary proteome profiles of an early pregnancy stage and non-pregnant sows and also reports differentially regulated proteins in saliva of early pregnant sows. Although some of the proteins were differentially expressed with higher fold change, their role during pregnancy has not been established yet in any species. Based on their recognized role in pregnancy and related events in different species, few proteins that are identified and found statistically differentially expressed in our study, such as thioredoxin, HSPA1A, alpha 1-S haptoglobin and glutathione S-transferase pi 1 may have potential as biomarkers for early pregnancy in pigs. Nevertheless, further research is required to evaluate their specific role during early pregnancy and validation in a large number of animals to establish them as biomarkers for early pregnancy diagnosis in pigs.

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