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Original Article

Proteomic analyses in fast and slow-cleaving bovine embryos – a search for potential markers of healthy pre-implantation embryos

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Abstract

Selection of embryos with potentially successful development is paramount to ensuring their viability and growth. While morphological assessment has been the mainstay of embryo selection, the identification of proteins that are expressed in the more reproductively successful fast-cleaving embryos as opposed to slow-cleaving ones may serve to reduce visual assessment of subjectivity in bovine. Through the use of proteomic mass spectrometric techniques, we found that 2387 proteins were commonly identified in both fast and slow-cleaving embryos, while 464 proteins were differentially identified. Slow-cleaving embryos showed a higher percentage of identified proteins involved in apoptotic processes, DNA repair and stress responses, while fast-cleaving bovine embryos showed a higher percentage of proteins identified that are involved in cell metabolism, gene transcription, and signal transduction. These differential protein expressions identified in the fast- and slow-cleaving embryos may serve as alternative potential markers of healthy pre-implantation embryos and improve overall bovine reproductive rate.

Keywords: proteomics, oocyte, bovine, embryo, blastocyst

1. Introduction

The advent of modern assisted reproduction techniques (ART) such as artificial insemination and *in vitro* fertilization (IVF) have greatly improved reproductive success rate in both the livestock industry and other mammals including human. Recent declines of the fertility rate in cattle (Khatib *et al.* 2009) has suggested that post-fertilization processes resulting in viable, pre-implantation embryos still present a significant bottleneck. Indeed, while fertilization rates in cattle has reached ≥90%, the calving rate is lower than 50% due to embryonic or fetal mortalities (Santos, Thatcher, Chebel, Cerri, & Galvao, 2004). Further information has revealed that >80% of IVF fertilized zygotes enter the early embryonic cleavage period (1-2-cell stage), but only 30-40% develop into blastocysts *in vitro* (Lonergan, Rizos, Gutierrez,

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Adan, Fair, & Boland, 2003). Therefore, an accurate determination of the quality of early cleaving embryos is an important step towards improving the success rate of ART in bovines.

The morphological evaluation of harvested embryos according to the criteria published by the International Embryo Transfer Society (IETS) (Perkel, Tscherner, Merrill, Lamarre, & Madan, 2015) has been the mainstay in predicting which embryos exert the highest implantation potential (Mermillod, Massip, & Dessy, 2003). The criteria involve metrics of the cleavage rates (cell number), cellular fragmentation, symmetry and multinucleation of blastomeres, along with zona pellucida thickness (Perkel *et al.* 2015; Rocha *et al.* 2016). Nevertheless, morphological evaluation remains highly subjective due the variations in the grading schemes and implementation of scoring systems (Mermillod *et al.* 2003; Perkel *et al.* 2015). Other proposed non-invasive techniques in determining embryo quality have been the study of the metabolomes and secretomes of individual embryos. However, the observation of the metabolome and secretome are often limited by difficulties in tracking the uptake/ secretion of trace metabolic substrates in the medium and the low detectable levels of secreted proteins, respectively (Krisher, Schoolcraft, & Katz-Jaffe, 2015; Perkel *et al.* 2015). Apart from the aforementioned criteria, the timing of the first zygotic cleavage in faster cleaving embryos have been shown to collaborate with a higher chance of survival, successful blastocyst development, implantation and ultimately pregnancies (Dinnyés, Lonergan, Fair, Boland, & Yang, 1999; Lechniak, Pers-Kamczyc, & Pawlak, 2008). In the present study, we demonstrated the differential proteomic profiles of slow- and fast-cleaving embryos and compared the marked changes of the identified proteins that are unique in these types of embryos. The findings from this study may provide a better understanding of the expression of specific proteins during early embryonic development which in turn serves as alternative potential markers for predicting successful rates of IVF in livestock.

2. Material and Methods

2.1 Chemicals

All chemicals in this study were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Media were prepared fresh weekly, filtered (0.2 µm, Sartorius, Minisart, CA, USA) and kept in sterile bottles. The aspirated cumulus oocyte complexes which had at least five layers of cumulus cells and homogeneous cytoplasm were selected for use in this study.

2.2 In vitro maturation of oocytes, fertilization, and embryo culturing

Since the ovaries used in this experiment (in year 2011) were harvested after sacrifices of the animals in the Pathumthani slaughterhouse, Pathumthani province, Thailand (in total 120 ovaries), ethic approval was not applicable. The collected ovaries were stored and and washed three times in physiological saline within 3 hours. Cumulus oocyte complexes (COCs) were collected by aspirating 2-8 mm follicles with an 18-gauge needle. To generate MII oocytes, COCs were washed three times in oocyte maturation medium (TCM 199) supplemented with 10% FCS, 0.2 mM pyruvate, 0.2 mM HEPES and 5µg/ml FSH and subjected to *in vitro* maturation (IVM). They were cultured in 50 µl drops of maturation medium in a humidified atmosphere of 5% CO_2 at 37 ℃. After culturing for 18-20 h, oocytes showing the first polar body were characterized as metaphase II. For IVF, sperm samples approximately 0.25 ml were placed under 1 ml TALP medium in a centrifugal tube and incubated at 5% CO_2 and 37 °C. After an hour of incubation, the upper part (500 µl) comprising mobile sperm were collected from the tubes and centrifuged at 2,300 × g for 10 min and resuspended in fertilization medium to a final concentration of 50 $\times 10^6$ cells/ml. Subsequently, a proportion of 10 µl of sperm suspension (5 $\times 10^5$ sperm) to 10 oocytes were incubated in a culture dish containing 40 µl of fertilization medium for 18-20 h at 5% CO_2 and 37 ℃. Thereafter, presumptive zygotes were denuded to remove cumulus cell and excess spermatozoa using TALP-HEPES, washed three times in synthetic oviductal fluid (SOF) and transferred to 100 µl droplets of SOF under mineral oil in petri dishes. These zygotes were incubated in 5% CO_2 and 37 ℃ane collected 48 h post-fertilization.

2.3 Protein extraction and SDS-PAGE

Thirty embryos of either the slow-cleaving or fast-cleaving types were added to one volume of 0.5% SDS, frozen on liquid nitrogen, ground, and sonicated in the warm water bath for 20 minutes. The lysate was centrifuged at 10,000 × g for 15 mins and the supernatant was transferred to a new tube, added with two volumes of cold acetone. The mixture was centrifuged at 10,000 × g for 15 min and the supernatant was removed. The pellet was dried and stored at -80 ° C prior to use.

The protein pellets obtained from each embryo were resuspended in 0.5% SDS and the protein concentration was determined by Lowry protein assay (Lowry, Rosebrough, Farr, & Randall, 1951). As the protein extracted from each type of embryo was unequal, for normalization, a total of 15 µg of protein from each type of embryo was run in each lane during 12.5% SDS-PAGE followed by silver staining for band visualization. Protein lanes in the gel were cut into 13 slices according to the size of the separated proteins, and each slice further cut into 1 mm^3 blocks.

2.4 In-gel digestion

The gel blocks were placed in 96-well plates, washed with 200 µl of milliQ water and shaken for 5 min. The gel blocks were dehydrated with 200 µl of 100% acetonitrile (ACN) and dried at room temperature for 10 min. Thereafter, all protein samples were subjected to 10 mM dithiothreitol (DTT) in 10 mM ammonium bicarbonate (NH_4HCO_3), incubated at room temperature for 1 h, removed, and a volume of 50 µl of 100 mM iodoacetamide (IAA) in 10 mM NH_4HCO_3 added to each well and incubated for 1 h. The samples were then washed twice with 200 ul of 100% ACN and digested by the addition of 10 ng of trypsin in 50% ACN in 10 mM NH_4HCO_3, followed by incubation at room temperature for 30 min. A volume of 30% ACN was further added to the protein samples and incubated at room

temperature overnight. The solution from each well was transferred to new 96 well plates. The original plate containing peptides were extracted twice by addition of 50% ACN in 0.1% formic acid (FA) and shaken for 10 min at room temperature. The latter solution was added to original solution followed by incubation at 40 °C until dry.

2.5 HCTUltra LC MS analysis

The HCTultra PTM Discovery System (Bruker Daltonics Ltd., Germany) coupled to an UltiMate 3000 LC System (Dionex Ltd., U.K.) was used to analyze the peptide solution. The eluents used in the system were: Eluent A - 0.1% formic acid and eluent B -80% acetonitrile in water containing 0.1% formic acid. Peptide separation was carried out with a linear gradient from 10% to 70% B for 13 min at a flow rate of 300 nl/min, including a regeneration step at 90% B and an equilibration step at 10% B. Peptide fragment mass spectra were acquired in data-dependent Auto MS mode with a scan range of 300−1500 m/z, 3 averages, and up to 5 precursor ions selected from the MS scan 50−3000 m/z.

2.6 Proteins quantitation and identification

DeCyder MS Differential Analysis software (DeCyderMS, GE Healthcare) was carried out for protein quantitation. The analyzed MS/MS data from DeCyderMS were submitted to database search using the Mascot software (Matrix Science, London, UK, (Perkins, Pappin, Creasy, & Cottrell, 1999). The data were searched against the NCBI database for protein identification. Proteins considered as identified proteins had at least one peptide with an individual mascot score corresponding to $p<0.05$. All protein IPI accession numbers were submitted into Uniprot accession number prior to gene ontology analysis. The GO analysis was carried out by a GO tool with clusters protein according to term provided by the GO project.

3. Results

3.1 Separation of fast- and slow-cleaving embryos, and fast and slow developing blastocysts

After performing *in vitro* fertilization (IVF), the fertilized oocytes were incubated for a further 48 hours to obtain early cleaving embryos. As shown in Figure 1, early embryos could be separated into two distinct groups of embryos, with slow-cleaving embryos displaying only 2- to 4-cells and the fast-cleaving embryos having 8-16 blastomeres with a certain number showing blastomeric compaction. To further elucidate the healthiness of developing embryos, they were incubated for a further six days post-IVF to observe the quality of blastocyst development. It was apparent that approximately 80% of the fast-cleaving embryos could further develop into blastocysts (exhibiting a clear formation of the blastocyst cavity, and the formation of the outer trophoectoderm (TE) layer and inner cell mass (ICM)) within a six-day of incubation period. On the other hand, most of the slow-cleaving embryos developed into blastocysts and underwent blastomeric compaction with or without blastocystic microlumens after an eight to nine-day of incubation period. The results suggested that fast developing embryos (within 48 h) had a higher propensity to undergo cell proliferation/differentiation due to better cellular metabolism and hence their observed activities. Therefore, we chose to further perform comparative proteomics analyses between these fast- and slow- cleaving embryos.

3.2 Proteomic and gene ontology analyses of the fast- and slow-cleaving embryos

The results from liquid chromatography mass spectrometry (LC-MS) (Figure 2) show that there were 2,387 proteins identified in both types of embryos. However, 247 proteins were uniquely identified in fast-cleaving embryos, while 217 proteins in slow-cleaving ones. The details and biological functions of the unique proteins in slow-cleaving embryos are shown in Table 1, while those of fast-cleaving embryos are shown in the Table 2. When only counting the proteins that obtained an ID score of over 7.0, only 105 and 99 uniquely identified proteins remained for the fast- and slow-cleaving embryos, respectively.

According to gene ontology analyses, the fast- and slow-cleaving embryos showed marked differences in the number of uniquely identified proteins when grouped according to their biological functions. As shown in Figures 3A and B, slow-cleaving embryos showed a higher percentage of proteins involved in apoptosis (5.07% vs. 0.40%) and cell adhesion (5.07% vs. 2.43%) from the total number of uniquely identified proteins (217) when compared to fast-cleaving embryos. In contrast, the fast-cleaving embryos showed a higher percentage of proteins involved in metabolism (11.34% vs. 8.76%), development (11.74% vs. 10.60%), DNA repair (1.21% vs. 0.92%), and signal transduction (12.15% vs. 9.68%).

However, when only unique proteins with ID scores above 7.0 were considered for determining the percentages of the grouping of proteins according to their biological functions, some marked changes between the two groups could be iterated (Figures 3C and D). Generally, the greater difference in the percentages of the biological functions between fast- and slow-cleaving embryo was noted. This threshold cutting caused the fast-cleaving embryos to have a much higher percentage of proteins involved in metabolism (9.52% vs 6.06%), transcription (12.38% vs. 8.2%), signal transduction (12.38% vs. 4.04%) and cell adhesion (5.71% vs 3.03%). On the other hand, slow-cleaving embryos showed higher percentages of proteins involved in apoptosis (5.05% vs 0.00%), DNA repair (2.02% vs. 0.00%) and the identification of the signal transduction/stress response protein

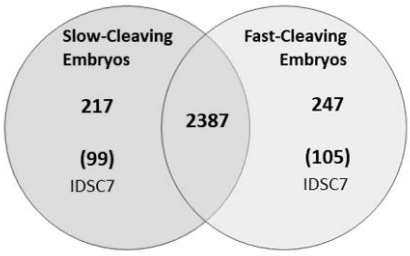

Figure 2. Venn diagram showing the number of uniquely and mutually identified proteins by LC-MS in fast- and slow-cleaving embryos

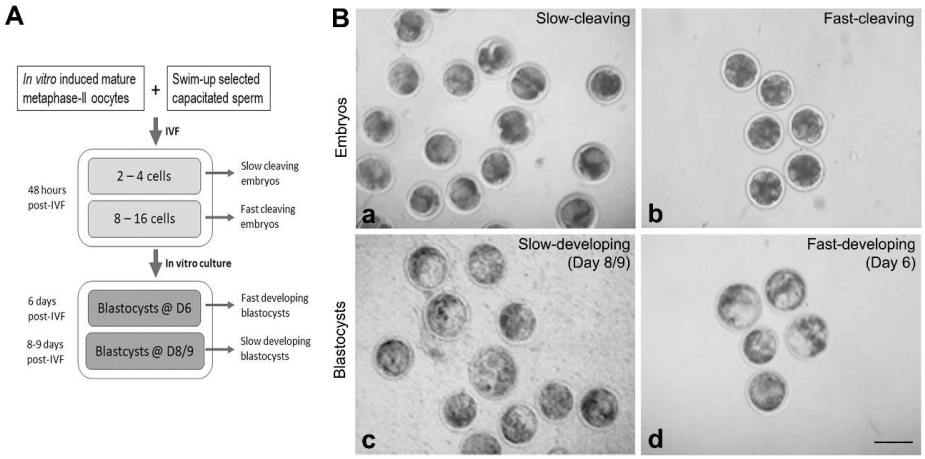

Figure 1. Diagram of oocyte maturation and in vitro fertilization to obtain embryos and blastocysts (A) and the microscopic examination of fast- and slow-cleaving embryos and fast- and slow- developing blastocysts (B). Bar = 100 µm.

Table 1. The top selected unique proteins in slow-cleaving embryos listed based on their biological process and protein ID scores > 7.0

Protein name and Species	Database	ID score	Sequence	Biological function
Nucleolar and coiled-body phosphoprotein 1 [Rattus norvegicus]	gi|12408334	10.28	KSVGAQSPK	apoptosis
KIAA1077 protein [Homo sapiens]	gi|5689491	9.11	HDEGHK	apoptosis
Izumo sperm-egg fusion protein 1 precursor [Mus musculus]	gi|65301151	14.49	KASQADFNSDYSGDK	cell adhesion
Lysozyme-like 6-like [Oryctolagus cuniculus]	gi|291406297	7.68	IVSGSR	cell adhesion
NAD(P) transhydrogenase [Homo sapiens]	gi|1000704	22.62	NIPQGAPVK	metabolic process
GPI inositol-deacylase-like [Monodelphis domestica]	gi|334330027	12.62	LIPDPCK	metabolic process
ATP-dependent DNA helicase PIF1-like [Ailuropoda melanoleuca]	gi|301756895	13.81	GSQPPK	DNA repair
Bloom syndrome protein [Bos taurus]	gi|119913638	12.22	KLILDR	DNA repair
Heat shock protein beta-1 [Canis lupus familiaris]	gi|50979116	66.43	LFDQAFGLPR	signal transduction
Glutamate receptor, metabotropic 8 [Homo sapiens]	gi|51095079	17.74	KSVTAPK	signal transduction

Table 2. The top selected unique proteins in fast-cleaving embryos listed based on their biological process and protein scores >7.0

Protein name and Species	Database	ID score	Sequence	Biological function
Keratocan-like [Ornithorhynchus anatinus]	gi|149637992	28.1	VPRISPR	cell adhesion
Protocadherin-11 X-linked-like, partial [Ailuropoda melanoleuca]	gi|301788426	14.32	CVVTDLGLHRLIVK	cell adhesion
Pappalysin 2 [Oryctolagus cuniculus]	gi|291397278	29.04	ATVVTGHSR	development
Protein shisa-2 homolog precursor [Macaca mulatta]	gi|302565436	16.83	QQGAGEPGR	development
Sulfotransferase family cytosolic 1B member 1-like [Monodelphis domestica]	gi|126330586	14.06	DAIFNR	metabolic process
Olfactory receptor Olr1203-like [Oryctolagus cuniculus]	gi|291383685	12.76	DVHVALSK	signal transduction
Rho GTPase-activating protein 22-like [Ailuropoda melanoleuca]	gi|301779109	12.78	TSPTGLGSQGSPAATSPGK	signal transduction
T complex responder [Mus musculus]	gi|227484	19.99	MLSDGRTIITFPNGTR	transcription
Niban-like protein 1 isoform 2 [Homo sapiens]	gi|79750824	14.34	YDYDSSSVR	transcription

heat shock protein beta 1 (HspB1 or Hsp27, Table 2). Therefore, the use of the ID score threshold of 7.0 filtered more than half of the unique proteins due to low ID scores in both types of embryos, while retaining those with relatively high ID scores, and therefore, providing increased confidence that the identified unique proteins were indeed expressed. The unique proteins of the fast- and slow-cleaving embryos which are grouped according to their biological functions and sorted according to their ID scores can be referred to in Tables 1 and 2.

4. Discussion

It has been generally accepted that higher quality and faster developing blastocysts contribute significantly to successful implantation and gestation in mammals (Perkel *et al.* 2015). In this study, using the more restrictive protein ID

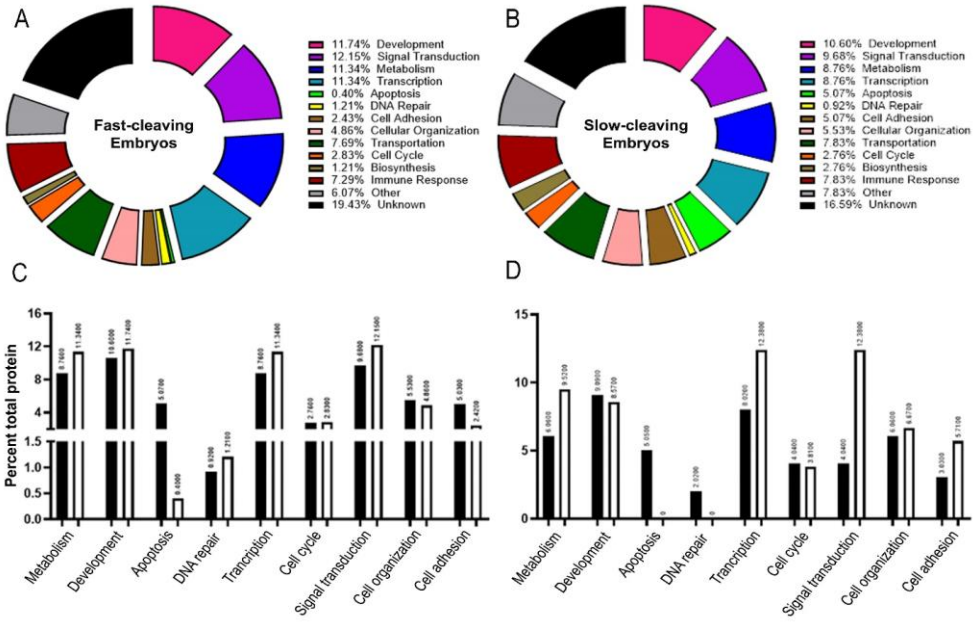

Figure 3. Graphs showing the difference in the percentages of uniquely identified proteins by LC-MS in the fast- and slow-cleaving embryos. Comparison of percentages of uniquely identified proteins in total using pie graphs, which includes proteins with "other" and "unknown" functions, in fast- (A) and slow-cleaving embryos (B). Comparison of percentages of uniquely identified proteins, not including "other" and "unknown" functions, in which the data are either non-filtered (C) or filtered with ID scores above 7.0 (D) in the fast- and slow-cleaving embryos (white and black bars, respectively).

score of more than 7 (IDSC7) revealed that slow-cleaving embryos differentially expressed more proteins associated with apoptosis and DNA repair when compared to fast-cleaving embryos. It is likely that these proteins may contribute to cells entering apoptosis indirectly rather than directly, as early-cleaving bovine zygotes up to the 8-cell stage have been shown to not display DNA damage associated with apoptosis. Apoptotic signs are mainly seen after the fourth cleavage cycle or in inner cell mass cells of blastocysts (Vandaele, Mateusen, Maes, de Kruif, & Van Soom, 2006). As for DNA damage, higher expression levels of nucleolar and coiled-body phosphoprotein 1 (NOLC1) decrease the level of telomeric repeat-binding factor 2 (TRF2) that protect telomeres, resulting in the DNA damage response that ultimately promotes apoptosis and cell proliferation arrest (Yuan, Xu, Li, & Tong, 2018).

Additionally, KIAA1077 or human sulfatase 1 (SULF1) and filamin-A-interacting protein 1-like (FILIP1) receptor both contribute to the decrease of cellular proliferation and promotes apoptosis in cancer cell lines (Reimann *et al.* 2020), while the kinase activity of receptor-interacting serine-threonine kinase 1 (RIPK1) by TNFR1 signaling induces caspase-8 mediated apoptosis (Newton, 2015). Taken all together, the expression of these proteins indicates that the machinery or factors contributing to future apoptosis have already been laid down during the early-cleaving stage of unhealthy embryos, thus, delaying entry into blastocyst stage. Moreover, the expression of proteins involved with DNA repair further indicated that slow-cleaving embryos may have higher DNA damage resulting from possibly paternal/maternal DNA damage or reactive oxygen species (ROS) (Ménézo, Dale, & Cohen, 2010). Furthermore, the expression of the DNA helicases PIF-1-like and Bloom syndrome protein has been reported to indicate the cellular response to DNA damage (Bochman, Sabouri, & Zakian, 2010; Tran *et al.* 2017). Accordingly, it has been reported that DNA damage occurs more frequently in slow-cleaving embryos (Bohrer, Coutinho, Duggavathi, & Bordignon, 2015; Dicks *et al.* 2017).

Fast-cleaving embryos uniquely expressed proteins involved in the advancement of blastocyst development such as pappalysin 2 (PAPP-A2; Table 2). PAPP-A2 cleaves IGFBP-5 and liberates IGF-I (Overgaard, Boldt, Laursen, Sottrup-Jensen, & Conover, 2001). This liberation of IGF-I is crucial as IGF-I produced in bovines has been shown to increase the proportion of developing zygotes into blastocysts (Makarevich & Markkula 2002; Neira, Tainturier, Pena, & Martal, 2010). Furthermore, IGF-I has been shown to have a protective effect on bovine embryos undergoing heat stress and apoptotic induction (Jousan & Hansen 2004).

Concerning metabolism, early cleaving embryos demonstrate a low level of metabolism using oxygen as a measurement. Upon entering the compaction (morula) to blastocyst phase, oxygen requirements drastically increase. With such increases of metabolism and oxygen use, an inevitable increase in ROS occurs, thus, dictating the expression of detoxification/scavenging proteins (Lonergan, Fair, Corcoran, & Evans, 2006; Perkel *et al.* 2015; Rocha *et al.* 2016). Proteins identified in the fast-cleaving embryos involved in metabolic process include both glutathione S-transferase A5 (GSTA5) and sulfotransferase family cytosolic 1B member 1-like (SULT1B1) (Table 2). Glutathione S-transferase (GST) are over-expressed by submission to pro-oxidants found in mammals (Meikle, Hayes, & Walker, 1992). Intracellular GST and GSH levels during *in vitro* maturation, fertilization and early embryo development are regarded a

marker of oocyte quality and the potential of embryo development after IVF (Zuelke, Jeffay, Zucker, & Perreault, 2003).

SULT1B1 is the member of the SULT subfamily and identified as a dopa/tyrosine sulfotransferase (Sakakibara *et al.* 1995). SULTs have a wide tissue distribution and function as a crucial detoxification enzyme system in adults and the developing human fetuses. (Falany, 1997; Negishi *et al.* 2001). As aforementioned, increased metabolism results in increased ROS production that can promote apoptosis, autophagocytosis, or necrosis (Covarrubias, Hernández-García, Schnabel, Salas-Vidal, & Castro-Obregón, 2008). Thus, GSTA5 and SULT1B1 may be crucial in embryo viability and reproductive potential by reducing intracellular ROS.

Lastly, cell adhesion proteins are crucial for embryogenesis after the onset of polarization and compaction during the 8-cell stage. Following the formation of apical and basolateral domains, compaction begins in which E-cadherin mediates the formation of intracellular basolateral contacts resulting in radial polarization. Accordingly, an increase in expression cell adhesion proteins in fast-cleaving embryos, in particular protocadherin-11-x-linked like (Pdch11x), may reflect better development. Protocadherins are involved in homophilic cell-cell aggregation and sorting during embryogenesis, in tissue separation during gastrulation and in neurogenesis (Zhang *et al.*, 2014). Furthermore, the keratocan-like proteoglycan is crucial in cellular aggregation and development of the corneal layer. (Liu, Birk, Hassell, Kane, & Kao, 2003). In contrast, the Izumo sperm-egg fusion protein 1 is involved in gamete fusion not embryogenesis, while its retention in cleaving embryos indicate the uncompleted ZGA process (Rubinstein, Ziyyat, Wolf, Le Naour, & Boucheix, 2003). In all, the unique proteins that were differentially expressed by the fast- and slow-cleaving embryos in this study may serve as alternative markers of quality of pre-implantation bovine embryos.

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