Characterization of exosomal release in bovine endometrial intercaruncular stromal cells

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Abstract

Background: Cell-to-cell communication between the blastocyst and endometrium is critical for implantation. In recent years, evidence has emerged from studies in humans and several other animal species that exosomes are secreted from the endometrium and trophoblast cells and may play an important role in cell-to-cell communication maternal-fetal interface during early pregnancy. Exosomes are stable extracellular lipid bilayer vesicles that encapsulate proteins, miRNAs, and mRNAs, with the ability to deliver their cargo to near and distant sites, altering cellular function(s). Furthermore, the exosomal cargo can be altered in response to environmental cues (e.g. hypoxia). The current study aims to develop an in vitro system to evaluate maternal-embryo interactions via exosomes (and exosomal cargo) produced by bovine endometrial stromal cells (ICAR) using hypoxia as a known stimulus associated with the release of exosomes and alterations to biological responses (e.g. cell proliferation).

Methods: ICAR cells cultured under 8 % O2 or 1 % O2 for 48 h and changes in cell function (i.e. migration, proliferation and apoptosis) were evaluated. Exosome release was determined following the isolation (via differential centrifugation) and characterization of exosomes from ICAR cell-conditioned media. Exosomal proteomic content was evaluated by mass spectrometry.

Results: Under hypoxic conditions (i.e. 1 % O2), ICAR cell migration and proliferation was decreased (~20 and ~32 %, respectively) and apoptotic protein caspase-3 activation was increased (~1.6 fold). Hypoxia increased exosome number by ~3.6 fold compared with culture at 8 % O2. Mass spectrometry analysis identified 128 proteins unique to exosomes of ICAR cultured at 1 % O2 compared with only 46 proteins unique to those of ICAR cultured at 8 % O2. Differential production of proteins associated with specific biological processes and molecular functions were identified, most notably ADAM10, pantetheinase and kininogen 2.

Conclusions: In summary, we have shown that a stimulus such as hypoxia can alter both the cellular function and exosome release of ICAR cells. Alterations to exosome release and exosomal content in response to stimuli may play a crucial role in maternal-fetal crosstalk and could also affect placental development.

Keywords: Bovine, Intercaruncular, Hypoxia, Exosomes

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Background
In dairy cattle, the average gestation length is approximately 282 days. The placenta is epitheliochorial, cotyledonary and non-deciduate [1]. Placentation is restricted to the aglandular maternal caruncles, where the fetal cotyledons come into contact with each other [2, 3]. They then form the placentome for maternal-fetal exchange of oxygen, nutrients and waste products. The glandular intercaruncular regions are associated with preserving the uterus in a state of quiescence and allowing a progressive uterine hypertrophy to accommodate the increasing needs of the growing feto-placental unit [4]. The uterine glands present in the intercaruncular endometrial areas secrete and release histotroph that is crucial for conceptus survival and growth [5] and is transported into the fetal circulation via the placental areolae. The establishment of a successful pregnancy requires the interactions between the endometrial cells and the early conceptus during maternal recognition of pregnancy [6, 7].

Cells located within intercaruncular region and associated with maternal fetal crosstalk include cells of stroma (intercaruncular stromal cell; ICAR) and epithelial origin. Both cell types are known to produce prostanoids (e.g. PGF$_{2\alpha}$) and have immunomodulatory functions [8, 9]. Interactions between these cells may also play a pivotal role in endometrial receptivity during early pregnancy as was reported in a co-culture study that human endometrial stromal cells can mediate epithelial cell function by promoting differentiation and inhibiting proliferation of endometrial epithelial cells [10]. In the bovine, endometrial stromal cells (as utilized in the current study) are known to differentially regulate the production of prostanoids and enzymes related to the production of prostanoids, in response to specific stimuli (e.g. inflammatory mediators and interferon tau) [8, 11]. ICAR cells were a kind gift from Professor Michel A. Fortier (Université Laval, Québec). ICAR cells are a well characterized bovine endometrial intercaruncular stromal cell line (ICAR cells) was utilized for the current study [8, 30]. ICAR cells were a kind gift from Professor Michel A. Fortier (Université Laval, Québec). ICAR cells were maintained in 175 cm$^2$ (T175, Corning Costar) culture flasks supplemented with exosome-free media (1640 Roswell Park Memorial Institute (RPMI) medium (Invitrogen, Life Technologies) with 10% heat-inactivated fetal bovine serum (Bovogen, Interpath services Pty Ltd) depleted of exosomes by ultracentrifugation (100,000 g for 20 h at 4 °C) and 1000 U/mL antibiotic-antimycotic solution (Gibco, Life Technologies) in a humidified cell culture incubator at 37 °C under an atmosphere of 5% CO$_2$-balanced N$_2$ to obtain a hypoxic (1% O$_2$) environment or under physiologically relevant conditions (8% O$_2$). Lactate dehydrogenase (LDH) assay was also
performed accordingly to the manufacturer’s protocol using the commercially available kit Pierce LDH cytotoxicity assay kit (Thermo scientific) to measure LDH in supernatants of ICAR cells cultured at 8 % O_2 and 1 % O_2 and ICAR cell viability was accessed. No significant difference in the LDH activity was observed (data not shown) between 8 % O_2 and 1 % O_2, indicating that the viability of ICAR cells was not affected by experimental condition.

**Cell migration assay**

The effect of oxygen tension on cell migration was assessed using methods as previously published [31]. Briefly, ICAR cells were plated (30,000 cells per well) and grown to confluence in a 96-well culture plate (Corning Costar) at 1 % O_2 or 8 % O_2 oxygen tension and a wound scratch was made on confluent monolayers using a 96-pin WoundMaker (Essen BioScience). Migration assays were performed in the presence of Mitomycin C (100 ng/mL, Sigma–Aldrich) to minimize any confounding effects of cell proliferation. The wound images were automatically acquired every 2 h for 48 h and registered by the IncuCyte software system (Essen BioScience). Data are presented as the Relative Wound Density (RWD, Eizen, v1.0 algorithm). RWD is a representation of the spatial cell density in the wound area relative to the spatial cell density outside of the wound area at every time point (time-curve).

**Cell proliferation assay**

Proliferation of ICAR cells was assessed using methods as previously published [28, 31]. In brief, the effect of oxygen tension on ICAR cell proliferation was assessed using a non-labelled cell monolayer confluence approach with a high density phase contrast real-time cell imaging system (IncuCyte™). ICAR cells were seeded at 40,000 cells per well in a 12-well culture plate (Corning Costar) and exposed to oxygen tension at 1 % O_2 or 8 % O_2 and the cell confluence (as the proliferation parameter) was measured at 0, 24 and 48 h.

**Cell apoptosis assay**

To assess the effect of hypoxia on cell apoptosis, ICAR cells were seeded at 5000 cells per well in 96-well culture plate (Corning Costar) in the presence of CellPlayer Kinetic Caspase-3/7 Apoptosis Assay Reagent (1:5000; Essen Biosciences) and imaged at 48 h with IncuCyte™. Cell apoptosis is determined by the measurement of the number of activated caspase 3/7 fluorescent objects count per mm2 divided by the percentage of cell confluence at 48 h (percentage of the area of field of view covered by cells with the metric ‘phase object confluence’) with the IncuCyte Zoom software using an integrated object counting algorithm.

**Exosome isolation from cell-conditioned media**

To study the effect of oxygen tension on exosome release, ICAR cells were incubated at 1 % O_2 or 8 % O_2 for 48 h. Exosomes were isolated from ICAR cell culture-conditioned media by successive differential centrifugation steps at 300 × g for 10 min and 2000 × g for 30 min. The supernatant was filtered through a 0.22-μm filter (Corning Costar) and ultracentrifuged at 100,000 × g for 20 h at 4 °C (Sorvall, SureSpin 630/360, Swinging-bucket ultracentrifuge rotor). Another round of ultracentrifugation washing steps was performed at 100,000 × g for 2 h at 4 °C (Beckman, Type 70.1 Ti, Fixed angle ultracentrifuge rotor). Exosomes were further enriched by layering on top of a discontinuous iodixanol gradient (OptiPrep, Sigma–Aldrich), which was centrifuged at 100,000 × g for 20 h (Beckman, Sw41Ti, Swinging-bucket ultracentrifuge rotor). Twelve fractions were obtained and diluted in 10 mL PBS (Gibco, Life Technologies). The fractions were washed with PBS and centrifuged at 100,000 × g for 2 h (Beckman, Type 70.1 Ti, Fixed angle ultracentrifuge rotor) and the exosomal pellets were suspended in 50 μL PBS.

**Nanoparticle Tracking Analysis (NTA)**

NTA measurements were performed using a NanoSight NS500 instrument (NanoSight NTA 3.0 Nanoparticle Tracking and Analysis Release Version Build 0064) as previously described [32, 33].

**Western blot analysis and transmission electron microscopy**

Exosomes were solubilized in RIPA buffer (Sigma–Aldrich) and separated by polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad) and probed with primary rabbit polyclonal antibody anti-CD63 (1:1000; EXOAB-CD63A-1, System Biosciences) and TSG101 (1:500; sc-6037, Santa Cruz Biotechnology). For electron microscopy analysis, exosome pellets were fixed in 3 % (w/v) glutaraldehyde and analyzed under an FEI Tecnai 12 transmission electron microscope (FEI, Hillsboro, Oregon, USA).

**Proteomic Analysis of Endometrial Exosomes by Mass Spectrometry (MS)**

Exosomes (10 μg of protein) were solubilized in RIPA buffer (Sigma–Aldrich) and separated by polyacrylamide gel electrophoresis. The gel was fixed in fixing solution (10:1:9; ethanol, acetic acid, MilliQ water respectively) for 15 min, washed in 1:1, ethanol and MilliQ water for 10 min and washed three times with MilliQ water. Proteins were stained with Coomassie Brilliant Blue R-250 staining solution (Bio-Rad) for 1 h and the gel was allowed to destain in MilliQ water until a clear background was obtained.
In-gel digestion methods for the mass spectrometric identification of exosomal proteins were performed by modification of previously published method [34]. In brief, each sample lane was cut into 24 gel slices and destained twice with 200 mM ammonium bicarbonate in 50 % acetonitrile solution for 45 min at 37 °C, desiccated using a vacuum centrifuge and then resuspended for 1 h at 65 °C. DTT in 20 mM dithiothreitol (DTT) in 25 mM ammonium bicarbonate solution and reduced for 1 h at 65 °C. DTT was then removed, and the samples were alkylated in 50 mM iodoacetamide and 25 mM ammonium bicarbonate at 37 °C in darkness for 40 min. Gel slices were washed three times for 45 min in 25 mM ammonium bicarbonate and then desiccated. Individual dried slices were then allowed to swell in 20 μL of 40 mM ammonium bicarbonate, 10 % acetonitrile containing 20 μg/mL trypsin (Sigma) for 1 h at room temperature. An additional 50 μL of the same solution was added and the samples were incubated overnight at 37 °C.

The supernatants were removed from the gel slices, and residual peptides were washed from the slices by incubating them three times in 50 μL of 0.1 % formic acid for 45 min at 37 °C. The original supernatant and washes were combined and desalted according to a modified version of the stage tip protocol that we have published [35, 36] using a 3-mm piece of an Empore C18 (Octadecyl) SPE Extraction Disk and the eluted peptides were dried in a vacuum centrifuge prior to spectral acquisition. The digested protein samples were analysed using the TripleTOF® 5600 mass spectrometer (ABSciex, Redwood City, CA) and Eksigent 1D+ NanoLC system with the chHiPLC system to obtain initial high mass accuracy survey MS/MS data, identifying the peptides present in the samples. The ChromXP C18-CL TRAP chHiPLC (200 μm × 6 mm, 3 μm, 120 Å) and analytical chHiPLC columns (200 μm × 15 cm; 3 μm, 120 Å) (Eksigent, Redwood City, CA) were used to separate the digested proteins. A 10 μL aliquot of digested material was injected onto the column and separated with a linear gradient of 5 to 10 % Buffer B for 2 min (Buffer A: 0.1 % Formic acid/water; Buffer B: acetonitrile/0.1 % formic acid), 10 to 40 % Buffer B (58 min), 40 to 50 % Buffer B (10 min), 50 to 95 % (10 min) with a flow rate of 500 nL/min. The column was flushed at 95 % buffer B for 15 min and re-equilibrated with 5 % Buffer B for 6 min. The in-depth proteomic analysis was performed using the Information Dependent Acquisition (IDA) experiments on the TripleTOF® 5600 System interfaced with a nanospray source. The source parameters were as follows: Cur gas at 25 psi, GS1 at 5 psi and IHT at 150 °C. A 250 msec accumulation time was set for the TOFMS survey scan and from this scan, the 10 most intense precursor ions were selected automatically for the MS/MS analysis (accumulation time of 150 msecs per MS/MS scan). Ions were isolated using unit resolution of the quadrupoles and rolling collision energy equation was used to calculate the collision energies of precursors. The precursor selection criteria included a minimum intensity of 50 counts per second (cps) and a charge state greater than 2 +.

Protein identification was determined using the ProteinPilot™ Software (v4.5 beta, AB Sciex, Redwood City, CA) with the Paragon algorithm. The search parameters were as follows: sample type, identification; cys alkylation, iodoacetamide; digestion, Trypsin; Instrument, TripleTOF 5600; special factors, none; and ID focus, biological modifications. The database was downloaded from the UniProt website in October 2015, which contained all proteins from Bos taurus. False discovery rate (FDR) was selected in the method and determined using a reversed sequence database. Data were subjected to ontology and pathway analysis using the protein analysis through evolutionary relationships tool (PANTHER) and gene ontology algorithms and classified based on biological process and molecular function categories [37].

Statistical analyses
The effects of oxygen tensions on ICAR cells are presented as mean ± SE for migration, proliferation and apoptosis assays (n = 6 independent experiments in duplicate). The number of exosomes is presented as number of particles per mL (mean ± SE, n = 3 independent isolations from 80 million cells each). The effects of oxygen tension on ICAR cells were identified by Student’s T tests (two-tailed) to compare the effect of hypoxia (i.e. 1 % O2) with the control group (i.e. 8 % O2) using a commercially-available software package (Prism 6, GraphPad Inc, La Jolla, CA 92037 USA).

Results
The Effect of Oxygen Tension on Bovine Endometrial (ICAR) cell migration and proliferation
The effect of normal oxygen tension (i.e. 8 % O2) and hypoxia (i.e. 1 % O2) on ICAR cell migration is presented in Fig. 1. ICAR cell migration was significantly lower under hypoxia compared with normal oxygen tension (Fig. 1a). Hypoxia decreased ICAR cell migration in a time-dependent manner (Fig. 1b). Area under the curve analysis indicated that hypoxia decreased ICAR cell migration by ~20 % compared with values observed at 8 % O2 (2173 ± 36 and 2620 ± 50 for 1 % O2 and 8 % O2, respectively) (Fig. 1c). Interestingly, hypoxia decreased ICAR cell proliferation in a time-dependent manner (Fig. 2a and b). Area under curve analysis showed that at 1 % O2, the proliferative capacity of ICAR cells was inhibited (p < 0.05) ~32 % compared with
Fig. 1 The effects of different oxygen tension on migration of bovine endometrial stromal cells (ICAR). 

**(a)** Graphical representation of the initial wound width (white) at 0 h and the area of the initial wound covered by advancing cells (grey) at 24 h and 48 h. Scale bar 300 μm. 

**(b)** Decreased ICAR cell migration under hypoxic conditions (1 % O\(_2\) (●)) compared with a normoxic 8 % O\(_2\) (○) over a period of 48 h. 

**(c)** Area under the curve analysis from (b); 8 % O\(_2\) (white bar) and 1 % O\(_2\) (black bar). Data are presented as mean ± SE, n = 6. In (b) and (c) \(P < 0.05\)

Fig. 2 The effects of different oxygen tension on proliferation of bovine endometrial stromal cells (ICAR). 

**(a)** Representative phase-contrast image of ICAR cells at 48 h when cultured under hypoxic conditions (1 % O\(_2\)) compared with a normoxic 8 % O\(_2\). Scale bar 200 μm. 

**(b)** Decreased \((p < 0.01)\) ICAR cell proliferation under hypoxic conditions (1 % O\(_2\) (●)) compared with a normoxic 8 % O\(_2\) (○) over a period of 48 h. 

**(c)** Area under the curve analysis from (b); 8 % O\(_2\) (white bar) and 1 % O\(_2\) (black bar). Data are presented as mean ± SE, n = 6. In (C) \(P < 0.05\)
cell proliferation at 8% O₂ (2.32 ± 0.18 and 3.41 ± 0.2 for 1% O₂ and 8% O₂, respectively) (Fig. 2c).

The Effect of Oxygen Tension on Bovine Endometrial (ICAR) cell apoptosis

The effect of oxygen tension on cell apoptosis is presented in Fig. 3. A hypoxic (1% O₂) environment altered cell morphology compared with cells cultured under normal conditions (8% O₂), displaying morphological hallmarks of apoptotic death (Fig. 3A,a and d). Fluorescent images acquired with IncuCyte™ (Fig. 3A, b and e) showed greater fluorescence in cells cultured under 1% O₂, indicating a higher activation of caspase-3/7 under hypoxic conditions compared with 8% O₂ (Fig. 3A, b and e). Apoptosis was quantified using the object counting algorithm in which the number of fluorescent objects was indicated with red x's in Fig. 3A (c and f). Quantification analysis showed that hypoxia increased (~1.6 fold) the apoptosis ratio (presented as activated caspase 3/7 fluorescent objects count per mm² divided by percentage of cell confluence at 48 h) compared with cells cultured under normal oxygen tension (Fig. 3B).

The Effect of Oxygen Tension on Exosome Release from Bovine Endometrial Cells (ICAR)

Exosomes were enriched by buoyant density gradient (see Material and Methods). We fractioned the 100,000 × g pelleted into 12 fractions and the Western blot analysis for TSG101 and CD63 showed positive protein abundance in fractions 1.17 and 1.18 g/mL (Fig. 4a). Exosomes were pooled between densities 1.16 and 1.18 g/mL. Morphology of exosomes was determined by electron microscopy (Fig. 4b), exosomes displayed a cup-shaped morphology with an estimated diameter of 100 nm. Hypoxia did not alter the size distribution of exosomes compared with normal oxygen tension (123 ± 2.7 nm versus 127 ± 1.7 nm for 8% O₂ and 1% O₂, respectively) (Fig. 4c). Interestingly,
hypoxia increased (~3.6 fold) the number of exosomes compared with values observed at normal oxygen tension (Fig. 4d).

**Proteomic Analysis of Bovine Endometrial ICAR-Derived Exosomes**

Mass spectrometric analysis identified over 250 exosomal proteins with 113 similar proteins identified as present in both exosomes of ICAR cultured at 1 % O₂ and at 8 % O₂. 128 proteins identified as unique to exosomes of ICAR cultured at 1 % O₂; 46 proteins were identified as unique to exosomes of ICAR cultured at 8 % O₂ (Table 1 A-C; Fig. 5a). Data were subjected to ontology and pathway analysis using PANTHER and gene ontology algorithms and classified based on biological process (Fig. 5b) and molecular function (Fig. 5c). In biological process, the clusters identified from individual proteins that are unique to and present only in exosomes of ICAR cultured at 1 % O₂ but not those at 8 % O₂ were: growth (0.7 %), locomotion (0.7 %) and reproduction (1.4 %) (Fig. 5b). In molecular functions, the proteins related to binding and catalytic activity were the greatest recognized in both exosomes of ICAR cultured at 1 % O₂ and to those of ICAR cultured at 8 % O₂ (Fig. 5c).

**Discussion**

A successful pregnancy is dependent of having a quality embryo and a receptive uterus synergizing with a synchronized crosstalk between the endometrium and embryo. Any insults or disturbances to its normal course can compromise implantation and the ability for the growing fetus to develop properly in the uterus [26]. The endometrium clearly has important functions in dairy cow pregnancy and we have now shown that exosomal release (30–120 nm) is part of its armamentarium which has analogous properties to similar tissues of other mammalian species.

In the present case, we have shown for the first time the effects of hypoxia on the biological activities of endometrial ICAR cells, including actions on the release and protein content of exosomes. Although it remains to be determined whether exosomes released from ICAR cells at different oxygen tensions also serve different functional goals, our data underscore that the content of exosomes may reflect the physiological state of the cells. Our non-exosomal characterization of the ICAR cells indicated that the migration and proliferative capacity of ICAR cells decreased, while activation of apoptotic caspase-3 was enhanced at 1 % O₂ (hypoxia), compared with an oxygen tension that was close to the bovine
| Protein ID   | Name                                      | Gene Name     | Biological Process (Total # Gene 69; Total #Function 146)                                                                 | Molecular function (Total # Gene 69; Total #Function 81)                                                                 |
|-------------|-------------------------------------------|---------------|-----------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------|
| A1LS23_BOVIN| Copine II (Fragment)                      | CPNE2         |                                                                                                                        |                                                                                                                        |
| A3KNS1_BOVIN| TSG101 protein                            | TSG101        | Metabolic process                                                                                                       | Catalytic activity                                                                                                    |
| A5D7L1_BOVIN| CLEC11A protein                           | CLEC11A       | Cellular process/Developmental process                                                                                | Binding/Structural molecule activity                                                                                   |
| A5D9D2_BOVIN| Complement component 4 binding protein, alpha chain | C4BPA        |                                                                                                                        |                                                                                                                        |
| A5P969_BOVIN| SERPINA10 protein                         | SERPINA10     | Biological regulation/Metabolic process                                                                               | Catalytic activity/Enzyme regulator activity                                                                         |
| A5PJE3_BOVIN| Fibrinogen alpha chain                    | FGA           |                                                                                                                        |                                                                                                                        |
| A5PK77_BOVIN| SERPINA11 protein                         | SERPINA11     | Biological regulation/Metabolic process                                                                               | Catalytic activity/Enzyme regulator activity                                                                         |
| A6QLB7_BOVIN| Adenyllyl cyclase-associated protein       | CAP1          |                                                                                                                        |                                                                                                                        |
| A6QLL8_BOVIN| Fructose-bisphosphate aldolase            | ALDOA         |                                                                                                                        |                                                                                                                        |
| A6QNZ7_BOVIN| Keratin 10 (Epidermolytic hyperkeratosis; keratosis palmaris et plantaris) | KRT10        |                                                                                                                        |                                                                                                                        |
| A6QPP2_BOVIN| SERPIND1 protein                          | SERPIND1      | Biological regulation/Metabolic process                                                                               | Catalytic activity/Enzyme regulator activity                                                                         |
| A6QPR1_BOVIN| PCYOX1 protein                            | PCYOX1        | Apoptotic process/Biological adhesion/Biological regulation/Cellular process/Developmental process/Immune system process/localization/Metabolic process | Catalytic activity/Receptor activity                                                                                   |
| B0JYQ6_BOVIN| Inter-alpha-trypsin inhibitor heavy chain H2 | ITIH2         |                                                                                                                        |                                                                                                                        |
| B0JYQ7_BOVIN| Inter-alpha-trypsin inhibitor heavy chain H1 | ITIH1         |                                                                                                                        |                                                                                                                        |
| C1QTNF3 protein | C1QTNF3                             | C1QTNF3       |                                                                                                                        |                                                                                                                        |
| LOC539596 protein | LOC539596                        | LOC539596     |                                                                                                                        |                                                                                                                        |
| CD63         |                                                                                          |               |                                                                                                                        |                                                                                                                        |
| AHSF         |                                                                                          |               |                                                                                                                        |                                                                                                                        |
| ALB          |                                                                                          |               |                                                                                                                        |                                                                                                                        |
| CSN151       |                                                                                          |               |                                                                                                                        |                                                                                                                        |
| WNT5A        |                                                                                          |               | Biological regulation/Cellular process/Developmental process/Multicellular organismal process/response to stimulus       | Binding                                                                                                               |
| SERPINA6     |                                                                                          |               | Biological regulation/Metabolic process                                                                               | Catalytic activity/Enzyme regulator activity                                                                         |
| FGB          |                                                                                          |               |                                                                                                                        |                                                                                                                        |
| ENO1         |                                                                                          |               |                                                                                                                        |                                                                                                                        |
| KRT14        |                                                                                          |               |                                                                                                                        |                                                                                                                        |
| QSOX1        |                                                                                          |               |                                                                                                                        |                                                                                                                        |
| AMBP         |                                                                                          |               |                                                                                                                        |                                                                                                                        |
| AMBP         |                                                                                          |               |                                                                                                                        |                                                                                                                        |
| ITGA3        |                                                                                          |               |                                                                                                                        |                                                                                                                        |
| ITIH2        |                                                                                          |               |                                                                                                                        |                                                                                                                        |
| SERPINC1     |                                                                                          |               |                                                                                                                        |                                                                                                                        |
Table 1 List of the common proteins identified in exosomes of ICAR cultured at 1 % O₂ and at 8 % O₂ (Continued)

| Accession | Protein Name | Function | Cellular/Metabolic Process | Activity |
|-----------|--------------|----------|----------------------------|----------|
| F1MTVS_BOVIN | Amino acid transporter | SLC1A5 | Cellular process/Metabolic process | Catalytic activity |
| F1MW44_BOVIN | Coagulation factor XIII A chain | F13A1 | Response to stimulus | |
| F1MXIS_BOVIN | IST1 homolog | IST1 | Developmental Process/Metabolic process | Enzyme regulator activity |
| F1MX6_BOVIN | Lactadherin | MFGE8 | Biological regulation/Metabolic process | Enzyme regulator activity |
| F1MY85_BOVIN | Complement C5a anaphylatoxin | C5 | Multicellular organismal process | |
| F1NO45_BOVIN | Complement component C7 | C7 | Response to stimulus | |
| F1RA1_BOVIN | Serine protease HTRA1 | HTRA1 | Metabolic process | |
| F1N116_BOVIN | Gelsolin | GSN | Immune system process/Metabolic process | Catalytic activity |
| F6QVC9_BOVIN | Annexin | ANXA5 | Developmental process/Metabolic process | Enzyme regulator activity |
| G3X6N3_BOVIN | Serotransferrin | TF | Response to stimulus | |
| G5E5A9_BOVIN | Fibronectin | FN1 | Multicellular organismal process | |
| Protein Name | Function | GO Terms | Activity |
|--------------|----------|----------|----------|
| ITIH3_BOVIN  | Inter-alpha-trypsin inhibitor heavy chain H3 | Biological regulation/Metabolic process | Binding/Catalytic activity/Enzyme regulator activity |
| ACTB_BOVIN   | Actin, cytoplasmic 1 | Structural molecule activity | structural molecule activity |
| ANXA6_BOVIN  | Annexin A6 | Metabolic process | Catalytic activity/Receptor activity/Transporter activity |
| CFAB_BOVIN   | Complement factor B | Biological adhesion/Cellular process/Immune system process/localization/Metabolic process/Response to stimulus | Catalytic activity/Receptor activity/Transporter activity |
| TBA1B_BOVIN  | Tubulin alpha-1B chain | Structural molecule activity | Structural molecule activity |
| LUM_BOVIN    | Lumican | Metabolic process | Receptor activity |
| UPAR_BOVIN   | Urokinase plasminogen activator surface receptor | Metabolic process | Catalytic activity |
| SNTD_BOVIN   | 5′-nucleotidase | Metabolic process | Catalytic activity |
| PGM1_BOVIN   | Phosphoglucomutase-1 | Metabolic process | Catalytic activity |
| Q09TE3_BOVIN | Insulin-like growth factor binding protein acid labile subunit | Metabolic process | Catalytic activity |
| Q17R18_BOVIN | Adenosine kinase | Metabolic process | Catalytic activity |
| FAS_BOVIN    | Coagulation factor V | Metabolic process | Catalytic activity |
| Q2KIF2_BOVIN | Leucine-rich alpha-2-glycoprotein 1 | Metabolic process | Receptor activity |
| CBP2_BOVIN   | Carboxypeptidase B2 | Metabolic process | Catalytic activity |
| Q2K47_BOVIN  | EH-domain containing 2 | Metabolic process | Catalytic activity |
| TBB5_BOVIN   | Tubulin beta-5 chain | Metabolic process | Catalytic activity |
| A1BG_BOVIN   | Alpha-1B-glycoprotein | Metabolic process | Receptor activity |
| HPT_BOVIN    | Haptoglobin | Metabolic process | Receptor activity |
| CO3_BOVIN    | Complement C3 | Metabolic process | Receptor activity |
| Q3MH8_BOVIN  | Alpha-amylase | Metabolic process | Catalytic activity |
| SAHH_BOVIN   | Adenosylhomocysteinase | Metabolic process | Catalytic activity |
| C09_BOVIN    | Complement component C9 | Metabolic process | Catalytic activity |
| Q3MH2_BOVIN  | F10 protein (Fragment) | Metabolic process | Catalytic activity |
| Q3MH20_BOVIN | FLOT1 protein (Fragment) | Metabolic process | Catalytic activity |
| Q3SYR0_BOVIN | Serpin peptidase inhibitor, clade A (Alpha-1 antiproteinase, antitrypsin), member 7 | Metabolic process | Catalytic activity |
Table 1 List of the common proteins identified in exosomes of ICAR cultured at 1 % O₂ and at 8 % O₂ (Continued)

| Protein ID     | Name                          | Gene Name       | Biological Process (Total # Gene 22; Total #Function 49)                     | Molecular function (Total # Gene 22; Total #Function 28) |
|----------------|-------------------------------|-----------------|--------------------------------------------------------------------------------|---------------------------------------------------------|
| FETA_BOVIN     | Alpha-fetoprotein             | AFP             | Developmental process/localization                                             |                                                         |
| Q3SZH5_BOVIN   | Angiotensinogen               | AGT             |                                                                                |                                                         |
| HEMO_BOVIN     | Hemopexin                     | HPX             | localization                                                                  |                                                         |
| Q3SZZ9_BOVIN   | FGG protein                   | FGG             |                                                                                |                                                         |
| PGK1_BOVIN     | Phosphoglycerate kinase 1     | PGK1            | Metabolic process                                                             | Catalytic activity                                      |
| Q3T101_BOVIN   | IGL@ protein                  | IGL@            |                                                                                |                                                         |
| G6PI_BOVIN     | Glucose-6-phosphate isomerase | GPI             | Metabolic process                                                             | Catalytic activity                                      |
| Q3ZBX0_BOVIN   | Basigin                       | BSG             |                                                                                |                                                         |
| Q3ZC87_BOVIN   | Pyruvate kinase (Fragment)    | PKM2            |                                                                                |                                                         |
| Q3ZC14_BOVIN   | 6-phosphogluconate dehydrogenase, decarboxylating | PGD | Metabolic process                                                             | Catalytic activity                                      |
| FETUB_BOVIN    | Fetuin-B                      | FETUB           |                                                                                |                                                         |
| EHD1_BOVIN     | EH domain-containing protein  | EHD1            | Biological regulation/Cellular process/localization/Metabolic process/Multicellular organismal process | Binding/Catalytic activity/Enzyme regulator activity |
| HPPD_BOVIN     | 4-hydroxyphenylypyruvate dioxygenase | HPD | Metabolic process                                                             | Catalytic activity                                      |
| QSEA67_BOVIN   | Inter-alpha (Globulin) inhibitor H4 (Plasma Kallikrein-sensitive glycoprotein) | ITIH4 |                                                                                |                                                         |
| QSGN72_BOVIN   | Alpha-1-acid glycoprotein     | agg             |                                                                                |                                                         |
| BHMT1_BOVIN    | Betaine–homocysteine S-methyltransferase 1 | BHMT | Cellular process/Metabolic process                                             | Catalytic activity                                      |
| Q5J801_BOVIN   | Endopin 2B                    |                 |                                                                                |                                                         |
| Q6T182_BOVIN   | Sex hormone-binding globulin (Fragment) | SHBG |                                                                                |                                                         |
| A2MG_BOVIN     | Alpha-2-macroglobulin         | A2M             | Biological regulation/Cellular process/Immune system process/Metabolic process/Response to stimulus | Binding/Catalytic activity/Enzyme regulator activity |
| PEDF_BOVIN     | Pigment epithelium-derived factor | SERPINF1 | Biological regulation/Metabolic process                                         | Catalytic activity/Enzyme regulator activity          |
| CHIA_BOVIN     | Acidic mammalian chitinase    | CHIA            | Immune system process/Metabolic process/Response to stimulus                  | Binding/Catalytic activity                             |
| IPSP_BOVIN     | Plasma serine protease inhibitor | SERPINA5 | Biological regulation/Metabolic process                                         | Catalytic activity/Enzyme regulator activity          |
| SPA31_BOVIN    | Serpin A3-1                   | SERPINA3-1      | Biological regulation/Metabolic process                                         | Catalytic activity/Enzyme regulator activity          |
| V6F9A2_BOVIN   | Apolipoprotein A-I preproprotein | APOA1 |                                                                                |                                                         |

B. List of 128 unique proteins identified in exosomes of ICAR cultured at 1 % O₂
Table 1  List of the common proteins identified in exosomes of ICAR cultured at 1 % O₂ and at 8 % O₂ (Continued)

| Protein ID      | Protein Name                                      | Biological process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
|-----------------|---------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| G1K1R6_BOVIN    | Galactokinase                                     | Apoptotic process/Biological regulation/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| G3P_BOVIN       | Glyceraldehyde-3-phosphate dehydrogenase          | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| Q0P5B0_BOVIN    | Arrestin domain containing 1                      | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| RL40_BOVIN      | Ubiquitin-60S ribosomal protein L40               | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| A5O9B6_BOVIN    | Syntenin                                          | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| Q8HZY1_BOVIN    | Serine protease inhibitor clade E member 2        | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| Q5E962_BOVIN    | Aldo-keto reductase family 1, member B1           | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| A7MBH9_BOVIN    | GNAI2 protein                                     | Biological regulation/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| GBB2_BOVIN      | Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 | Biological regulation/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| I6YIV1_BOVIN    | Annexin                                           | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| F16P1_BOVIN     | Fructose-1,6-bisphosphatase 1                      | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| F1N3Q7_BOVIN    | Apolipoprotein A-IV                                | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| AK1A1_BOVIN     | Alcohol dehydrogenase [NADP(+)i]                  | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| A5O7B4_BOVIN    | CPNE8 protein                                     | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| H590A_BOVIN     | Heat shock protein HSP 90-alpha                   | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| Q1JPA2_BOVIN    | Eukaryotic translation elongation factor 1 gamma (Fragment) | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| SERA_BOVIN      | D-3-phosphoglycerate dehydrogenase                 | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| Q3T08S_BOVIN    | OGN protein                                       | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| A80BT6_BOVIN    | Monocyte differentiation antigen CD14             | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| ASPK73_BOVIN    | Fructose-bisphosphate aldolase                    | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| G5E5U7_BOVIN    | S-adenosylmethionine synthase                     | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| F1N2W0_BOVIN    | Prostaglandin reductase 1                         | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| IF4A1_BOVIN     | Eukaryotic initiation factor 4A-1                  | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| Q05B55_BOVIN    | IGK protein                                       | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| F1N1D4_BOVIN    | Protein tweety homolog                            | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| A4F94_BOVIN     | KRT6A protein                                     | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| RGN_BOVIN       | Regucalcin                                        | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| I433E_BOVIN     | 14-3-3 protein epsilon                            | Metabolic process/Cellular component organization or biogenesis/Cellular process/Developmental process/Growth/localization/Metabolic process/Multicellular organismal process/Response to stimulus |
| Accession    | Description                                                                 | Gene   | Protein Name | Function                                                                                           | Activity               |
|-------------|------------------------------------------------------------------------------|--------|--------------|---------------------------------------------------------------------------------------------------|------------------------|
| Q2HJB6_BOVIN| Procollagen C-endopeptidase enhancer                                        | PCOLCE | Q2HJB6_BOVIN | Biological adhesion/Biological regulation/Cellular process/Developmental process/Immune system process/localization/Metabolic process/Multicellular organismal process/Response to stimulus | Binding/Catalytic activity/Enzyme regulator activity/Receptor activity/Transporter activity |
| B8YB76_BOVIN| Homogentisate 1,2-dioxygenase                                                  | HGD    | B8YB76_BOVIN | Metabolic process                                                                                   | Catalytic activity     |
| DHSO_BOVIN  | Sorbitol dehydrogenase                                                       | SORD   | DHSO_BOVIN   | Cellular component organization or biogenesis/Immune system process/Metabolic process/Response to stimulus | Transporter activity   |
| H571A_BOVIN  | Heat shock 70 kDa protein 1A                                                  | HSPA1A | H571A_BOVIN  | Metabolic process                                                                                   | Catalytic activity     |
| Q3ZBQ9_BOVIN| APOM protein                                                                  | APOM   | Q3ZBQ9_BOVIN | Metabolic process                                                                                   | Catalytic activity     |
| PYGL_BOVIN  | Glycogen phosphorylase, liver form                                            | PYGL   | PYGL_BOVIN   | Metabolic process                                                                                   | Catalytic activity     |
| A6QP30_BOVIN| CPN2 protein                                                                  | CPN2   | A6QP30_BOVIN | Cellular process/Multicellular organismal process                                                     | Receptor activity      |
| ARF3_BOVIN  | ADP-ribosylation factor 3                                                    | ARF3   | ARF3_BOVIN   | Cellular process/localization/Metabolic process                                                      | Binding/Catalytic activity |
| G3MYH4_BOVIN| Tetratspanin (Fragment)                                                       | CD81   | G3MYH4_BOVIN | Metabolic process                                                                                   | Structural molecule activity |
| ACTC_BOVIN  | Actin, alpha cardiac muscle 1                                                | ACTC1  | ACTC_BOVIN   | Cellular component organization or biogenesis/Cellular process/Developmental process/localization    | Structural molecule activity |
| GALM_BOVIN  | Aldose 1-epimerase                                                            | GALM   | GALM_BOVIN   | Metabolic process                                                                                   | Catalytic activity     |
| TSN6_BOVIN  | Tetraspanin-6                                                                | TSPAN6 | TSN6_BOVIN   | Metabolic process                                                                                   | Binding/Receptor activity |
| Q3ZC83_BOVIN| Solute carrier family 29 (Nucleoside transporters), member 1                 | SLC29A1| Q3ZC83_BOVIN | Metabolic process                                                                                   | Transporter activity   |
| B4GA1_BOVIN | Beta-1,4-glucuronyltransferase 1                                              | B4GAT1 | B4GA1_BOVIN  | Metabolic process                                                                                   | Catalytic activity     |
| ADA10_BOVIN | Disintegrin and metallopeptidase domain-containing protein 10               | ADAM10 | ADA10_BOVIN  | Apoptotic process/Developmental process/Reproduction                                                   |                        |
| A6QR28_BOVIN| Phosphoserine aminotransferase                                                | PSAT1  | A6QR28_BOVIN | Metabolic process                                                                                   | Catalytic activity     |
| Q1JR86_BOVIN| Acetyl-Coenzyme A acetyltransferase                                           | ACAT2  | Q1JR86_BOVIN | Metabolic process                                                                                   |                        |
| DDBX_BOVIN  | Dihydrodiol dehydrogenase 3                                                  | DDBX   | DDBX_BOVIN   | Metabolic process                                                                                   | Catalytic activity/Transporter activity |
| A7VE11_BOVIN| IGSF8 protein                                                                 | IGSF8  | A7VE11_BOVIN | Metabolic process                                                                                   | Catalytic activity     |
| F1MS32_BOVIN| Apolipoprotein D                                                             | APOD   | F1MS32_BOVIN | Metabolic process                                                                                   | Catalytic activity     |
| A6QP64_BOVIN| VPS37B protein (Fragment)                                                     | VPS37B | A6QP64_BOVIN | Metabolic process                                                                                   | Catalytic activity     |
| Q2KH4W4_BOVIN| Lecithin-cholesterol acyltransferase                                         | LCAT   | Q2KH4W4_BOVIN| Metabolic process                                                                                   | Catalytic activity     |
| GBB1_BOVIN  | Guanine nucleotide-binding protein G()G()G()G()T subunit beta-1               | GNB1   | GBB1_BOVIN   | Metabolic process                                                                                   | Binding/Catalytic activity |
| GNA11_BOVIN | Guanine nucleotide-binding protein subunit alpha-1                            | GNA11  | GNA11_BOVIN  | Metabolic process                                                                                   | Catalytic activity     |
| Q17K4_BOVIN | Epoxide hydrolase 2, cytoplasmatic                                            | EPHX2  | Q17K4_BOVIN  | Metabolic process                                                                                   | Catalytic activity     |
### Table 1 List of the common proteins identified in exosomes of ICAR cultured at 1 % O2 and at 8 % O2 (Continued)

| Protein ID     | Protein Name and Description                                                                 | Cellular Component or Biogenesis/Cellular Process | Metabolic Process/Response to Stimulus |
|----------------|---------------------------------------------------------------------------------------------|-------------------------------------------------|---------------------------------------|
| K2C7_BOVIN     | Keratin, type II cytoskeletal 7                                                            | Cellular component organization or biogenesis/Cellular process/Developmental process | Structural molecule activity          |
| CLIC1_BOVIN    | Chloride intracellular channel protein 1                                                     | Biological regulation/Cellular process/ Metabolic process/Response to stimulus | Binding/Catalytic activity/ Structural molecule activity/Translation regulator activity |
| Q08DW4_BOVIN   | Mannan-binding lectin serine peptidase 1 (C4/C2 activating component of C-reactive factor)  | MASP1                                            |                                       |
| B4GT1_BOVIN    | Beta-1,4-galactosyltransferase 1                                                             | BAGALT1                                          |                                       |
| ASD766_BOVIN   | Tetraspanin                                                                                  | CD82                                             | Celluar process/Response to stimulus  |
| ASD973_BOVIN   | Alpha isoform of regulatory subunit A, protein phosphatase 2                                 | PPP2R1A                                          | Binding/Receptor activity             |
| E1B726_BOVIN   | Plasminogen                                                                                  | PLG                                              |                                       |
| G5E69_BOVIN    | Histone H2B                                                                                  | LOC101904777                                    | Celluar component organization or biogenesis/Cellular process/Metabolic process |
| ADIPO_BOVIN    | Adiponectin                                                                                  | ADIPOQ                                           |                                       |
| F1MBCS_BOVIN   | Coagulation factor IX                                                                        | F9                                               |                                       |
| A2VDL2_BOVIN   | Solute carrier family 2 (Facilitated glucose transporter), member 3                         | SLC2A3                                           |                                       |
| VPS4B_BOVIN    | Vacuolar protein sorting-associated protein 4B                                               | VPS4B                                            |                                       |
| G3X8B1_BOVIN   | Peptidyl-prolyl cis-trans isomerase                                                          | LOC613401                                       |                                       |
| K4JB97_BOVIN   | Alpha-2-macroglobulin variant 4                                                             | A2M                                              |                                       |
| ACTG_BOVIN     | Actin, cytoplasmic 2                                                                         | ACTG1                                            | Celluar component organization or biogenesis/Cellular process/localization         |
| Q1JPG7_BOVIN   | Pyruvate kinase                                                                             | PKLR                                             | Structural molecule activity          |
| GTR1_BOVIN     | Solute carrier family 2, facilitated glucose transporter member 1                           | SLC2A1                                           |                                       |
| F1N342_BOVIN   | Protein tweety homolog                                                                       | TTYH2                                            | Localization                          |
| ADHX_BOVIN     | Alcohol dehydrogenase class-3                                                               | ADH5                                             | Metabolic process                     |
| URP2_BOVIN     | Fermitin family homolog 3                                                                   | FERMT3                                           | Transporter activity                  |
| E1B7N2_BOVIN   | Histone H4                                                                                   | HISTH4                                           | Catalytic activity                    |
| EF2_BOVIN      | Elongation factor 2                                                                          | EEF2                                             |                                       |
| KLKB1_BOVIN    | Plasma kallikrein                                                                           | KLKB1                                            | Biological regulation/localization/ | Binding/Transduction regulator activity |
| ESTD_BOVIN     | S-formylglutathione hydrolase                                                                | ESD                                              | Biological regulation/localization/Metabolic process/Response to stimulus | Binding/Catalytic activity/Enzyme regulator activity/Receptor activity |
| SEPR_BOVIN     | Prolyl endopeptidase FAP                                                                      | FAP                                              | Cellular process/Immune system process/localization/Metabolic process/Multicellular organismal process/Response to stimulus | Binding/Catalytic activity |
| QSEA54_BOVIN   | Solute carrier family 3 (Activators of dibasic and neutral amino acid transport), member 2   | SLC3A2                                           |                                       |
| ID       | Description                                                                 | GO Term                                | Functions                                                                                     |
|----------|-----------------------------------------------------------------------------|----------------------------------------|---------------------------------------------------------------------------------------------|
| Q1JP99   | G protein-coupled receptor, family C, group S, member B                      | GPRC8                                  | Cellular process, Receptor activity                                                        |
| F1M05    | Aconitate hydratase                                                         | ACO1                                   | Metabolic process, Catalytic activity                                                       |
| F1MJ12   | Complement C1s subcomponent                                                 | C1S                                    | Biological adhesion/Cellular process/Immune system process/localization/Response to stimulus |
| CNDP2    | Cytosolic non-specific dipeptidase                                           | CNDP2                                  | Metabolic process, Catalytic activity                                                       |
| Q2B5Q1   | Coagulation factor XIII, B polypeptide                                      | F13B                                   | Biological adhesion/Cellular process/Immune system process/localization/Response to stimulus |
| Q1J972   | Colony stimulating factor 1 receptor                                        | CSF1R                                  | Metabolic process, Catalytic activity                                                       |
| Q0V0D3   | CD44 antigen                                                                | CD44                                   | Metabolic process                                                                           |
| G3X6Y4   | Osteomodulin                                                               | OMD                                    | Metabolic process                                                                           |
| G3X6Y4   | Guanidinoacetate N-methyltransferase                                        | G3X6Y4                                 | Metabolic process                                                                           |
| WVA1     | von Willebrand factor A domain-containing protein 1                         | WVA1                                   | Metabolic process                                                                           |
| SERC3    | Serine incorporator 3                                                       | SERC3                                  | Metabolic process                                                                           |
| Q7OYV4   | Kappa-casein (Fragment)                                                     | csn3                                   | Metabolic process                                                                           |
| G3X6Q8   | Pentraxin-related protein PTX3                                              | G3X6Q8                                 | Metabolic process                                                                           |
| K7QEL2   | MHC class I antigen                                                         | K7QEL2                                 | Metabolic process                                                                           |
| TCPQ     | T-complex protein 1 subunit theta                                           | TCPQ                                   | Metabolic process                                                                           |
| F1N6Z9   | 26S proteasome regulatory subunit 5                                         | F1N6Z9                                 | Metabolic process                                                                           |
| PRS23    | Serine protease 23                                                          | PRS23                                  | Metabolic process                                                                           |
| P0Y7N1   | Cathepsin L2                                                                | P0Y7N1                                 | Metabolic process                                                                           |
| A4V799   | FCNB protein                                                                | A4V799                                 | Metabolic process                                                                           |
| A7YV37   | CDS8 protein (Fragment)                                                     | A7YV37                                 | Metabolic process                                                                           |
| F1MT5P   | WD repeat-containing protein 1                                              | F1MT5P                                 | Metabolic process                                                                           |
| A7E3DQ   | CCDC45 protein (Fragment)                                                   | A7E3DQ                                 | Metabolic process                                                                           |
| Q8C0K1   | Myeloid-associated differentiation marker                                   | Q8C0K1                                 | Metabolic process                                                                           |
| A1L570   | Ephrin-B1                                                                   | A1L570                                 | Metabolic process                                                                           |
Table 1 List of the common proteins identified in exosomes of ICAR cultured at 1 % O<sub>2</sub> and at 8 % O<sub>2</sub> (Continued)

| Protein ID       | Name                                      | Gene Name | Biological Process (Total # Gene 22; Total #Function) | Molecular function (Total # Gene 22; Total #Function) |
|------------------|-------------------------------------------|-----------|-------------------------------------------------------|-------------------------------------------------------|
| F1N049_BOVIN     | Actin-related protein 3 (Fragment)        | ACTR3     | Biological regulation/Metabolic process               | Catalytic activity/Enzyme regulator activity           |
| PAI1_BOVIN       | Plasminogen activator inhibitor 1         | SERPINE1  | Biological regulation/Immune system process/Multicellular organismal process |                |
| Q3ZC30_BOVIN     | Sulfotransferase                          | SULT1E1   |                                                        |                                                        |
| COL11_BOVIN      | Collectin-11                              | COLEC11   |                                                        |                                                        |
| MPZL1_BOVIN      | Myelin protein zero-like protein 1        | MPZL1     | Cellular process/localization                         | Transporter activity                                   |
| G5E595_BOVIN     | Lys-63-specific deubiquitinase BRCC36     | BRCC3     |                                                        |                                                        |
| O1897T_BOVIN     | Tenascin-X                                | TN-X      |                                                        |                                                        |
| A6H7D3_BOVIN     | KRT18 protein (Fragment)                  | KRT18     |                                                        |                                                        |
| J9ZG05_BOVIN     | Integrin alpha V subunit                  | ITGAV     |                                                        |                                                        |
| B0JYN3_BOVIN     | L-lactate dehydrogenase                   | LDHB      |                                                        |                                                        |
| MB211_BOVIN      | Protein mab-21-like 1                     | MAB21L1   |                                                        |                                                        |
| E1B7R4_BOVIN     | Eukaryotic translation initiation factor 3 subunit A | EIF3A     | Biological regulation/Metabolic process               | Binding/Translation regulator activity                  |
| C. List of 46 unique proteins identified in exosomes of ICAR cultured at 8 % O<sub>2</sub>

| Protein ID       | Name                                      | Gene Name | Biological Process (Total # Gene 22; Total #Function) | Molecular function (Total # Gene 22; Total #Function) |
|------------------|-------------------------------------------|-----------|-------------------------------------------------------|-------------------------------------------------------|
| F1MMD7_BOVIN     | Inter-alpha-trypsin inhibitor heavy chain H4 | ITIH4     |                                                        |                                                        |
| F1N3A1_BOVIN     | Thrombospondin-1                          | THBS1     |                                                        |                                                        |
| PLMN_BOVIN       | Plasminogen                               | PLG       |                                                        |                                                        |
| F1MYN5_BOVIN     | Fibulin-1                                 | FBLN1     |                                                        |                                                        |
| F1MMN5_BOVIN     | Kininogen-1                               | KNG1      |                                                        |                                                        |
| EF1A1_BOVIN      | Elongation factor 1-alpha 1               | EEF1A1    |                                                        |                                                        |
| ITAV_BOVIN       | Integrin alpha-V                          | ITGA5     |                                                        |                                                        |
| F1MK44_BOVIN     | Integrin alpha-5                          | ITGA5     |                                                        |                                                        |
| TTHY_BOVIN       | Transthyretin                             | TTR       | localization                                           | Transporter activity                                   |
| F1NC45_BOVIN     | Complement factor H (Fragment)            | CFH       |                                                        |                                                        |
| J9Q9Q7_BOVIN     | Periostin variant 9                       |           |                                                        |                                                        |
| ACT5_BOVIN       | Actin, alpha skeletal muscle              | ACTA1     |                                                        |                                                        |
| E1B9K1_BOVIN     | Polyubiquitin-C                           | UBC       |                                                        |                                                        |
| A7YWR0_BOVIN     | Apolipoprotein E                          | APOE      |                                                        |                                                        |
| FA9_BOVIN        | Coagulation factor IX                     | F9        | Apoptotic process/Biological regulation/Developmental process/Immune system process/localization/Metabolic process/Multicellular organismal process/Response to stimulus | Binding/Catalytic activity/Enzyme regulator activity/Receptor activity |
| COMP_BOVIN       | Cartilage oligomeric matrix protein       | COMP      |                                                        |                                                        |
Moreover, the effect on migration was greater when exposed at 1% O₂ [39]. Interestingly, no relationship between oxygen tension and cell proliferation and apoptosis was observed in this previous study. Differences in cell types may explain this observation. Ito et al.

### Table 1

| Accession | Description | Cell Type | Molecular Function | Biological Process |
|-----------|-------------|-----------|--------------------|--------------------|
| K2C80_BOVIN | Keratin, type II cytoskeletal 80 KRT80 | Cellular component organization or biogenesis/Cellular process/Developmental process | Structural molecule activity |
| TRFE_BOVIN | Serotransferrin TF | localization/Metabolic process | Catalytic activity |
| K4JDR8_BOVIN | Alpha-2-macroglobulin variant S A2M | Cellular component organization or biogenesis/Cellular process/Metabolic process | Binding |
| Q3P272_BOVIN | CP protein (Fragment) CP | Cellular process/localization | Transporter activity |
| J92W47_BOVIN | Integrin beta | Metabolic process/Response to stimulus | Binding/Receptor activity |
| F1MM86_BOVIN | Complement component C6 C6 | Biological regulation/Cellular process/Metabolic process | Binding/Catalytic activity |
| E1H02_BOVIN | Fibromodulin FMOD | Biological regulation/Metabolic process | Binding/Catalytic activity/Translation regulator activity |
| VNN1_BOVIN | Pantetheinase VNN1 | Cellular component organization or biogenesis/Cellular process/Developmental process | Structural molecule activity |
| G3X807_BOVIN | Histone H4 (Fragment) | Metabolic process/Response to stimulus | Transporter activity |
| MOT1_BOVIN | Monocarboxylate transporter 1 SLC16A1 | Cellular process/localization | Transporter activity |
| TF_BOVIN | Tissue factor F3 | Biological regulation/Cellular process/Response to stimulus | Binding/Receptor activity |
| HS71L_BOVIN | Heat shock 70 kDa protein 1-like HSPA1L | Metabolic process/Response to stimulus | Transporter activity |
| Q3ZCA7_BOVIN | Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3 GNAI3 | Metabolic process/Response to stimulus | Transporter activity |
| IDHC_BOVIN | Isocitrate dehydrogenase [NADP] cytoplasmic IDH1 | Metabolic process/Response to stimulus | Transporter activity |
| Q1PB8_C_BOVIN | CD14 (Fragment) | Biological regulation/Metabolic process | Binding/Catalytic activity |
| F1MJJ8_BOVIN | Radixin (Fragment) RDX | Localization | Transporter activity |
| IF4A2_BOVIN | Eukaryotic initiation factor 4A-II EIF4A2 | Biological regulation/Metabolic process | Binding/Catalytic activity/Translation regulator activity |
| C1QB_BOVIN | Complement C1q subcomponent subunit B C1QB | Metabolic process/Response to stimulus | Transporter activity |
| A6QPD4_BOVIN | LOC790886 protein LOC790886 | Metabolic process/Multicellularorganismal process | Transporter activity |
| CTL2_BOVIN | Choline transporter-like protein 2 SLC4A2 | Localization | Transporter activity |
| HPC11_BOVIN | Hippocalcin-like protein 1 HPCAL1 | Cellular process/Response to stimulus | Transporter activity |
| Q24K07_BOVIN | Vacuolar protein sorting 11 homolog (S. cerevisiae) VPS11 | Metabolic process/Response to stimulus | Transporter activity |
| Q5H9M6_BOVIN | Dynein heavy chain (Fragment) Bv2 | Metabolic process/Multicellularorganismal process | Transporter activity |
| Q864S1_BOVIN | Cathepsin C (Fragment) | Metabolic process/Multicellularorganismal process | Transporter activity |
| Q4ZJ50_BOVIN | MHC class I antigen (Fragment) BoLA-N | Metabolic process/Multicellularorganismal process | Transporter activity |
| Q58C24_BOVIN | Flotillin 2 FLOT2 | Metabolic process/Multicellularorganismal process | Transporter activity |
| MBL2_BOVIN | Mannose-binding protein C MBL | Binding | Transporter activity |
| TM214_BOVIN | Transmembrane protein 214 TMEM214 | Metabolic process/Response to stimulus | Transporter activity |
| Q8MIR1_BOVIN | Nicotinic acetylcholine receptor beta 2 subunit (Fragment) CHRNA2 | Metabolic process/Response to stimulus | Transporter activity |
| Q5E9W1_BOVIN | CDC45-like CDC45L | Metabolic process/Response to stimulus | Transporter activity |

Mass spectrometric (with a set FDR of 5%) identification of proteins was present in exosomes generated by ICAR cultured at 1% O₂ and at 8% O₂. Data were subjected to ontology and pathway analysis using PANTHER and gene ontology algorithms and classified based on biological process and molecular function.

endometrial physiological oxygen levels (8% O₂ [38]). Moreover, the effect on migration was greater when exposed at 1% O₂ [39]. Interestingly, no relationship between oxygen tension and cell proliferation and apoptosis was observed in this previous study. Differences in cell types may explain this observation. Ito et al.
described the rate of proliferation of human mesenchymal stem cell (MSCs) was observed to be highest in 5 % O₂ and the lowest in < 0.1 % O₂ conditions [40]. The MSCs at severely induced hypoxic conditions (< 0.1 % O₂), showed a decrease in proliferative ability, but were able to maintain viability for at least 48 h through increased glucose availability, to facilitate the generation of energy. Similar results were obtained from an airway smooth muscle study [41]. Hence, our cells have relatively normal proliferation responses to decreased oxygen tension.

Our study suggests that exosomes can serve as a vector for signaling molecules that harbor a variety of bioactive molecules including proteins at the conceptus-endometrial interface and that has the potential to modulate the functions of targeted cells during early pregnancy. Endometrial exosome release may also be modulated during an insult such as infection [42, 43].

In the current study we utilized hypoxia (i.e. 1 % O₂) as a known modulator of exosome release as documented by alteration to both the number of exosomes released as well as differences in the exosomal content (cargo) [24, 27, 29].

In our study, endometrial cells exposed to 1 % O₂-released ~3.6 more exosomes relative to the 8 % O₂ treatment, suggesting that hypoxia modulates cell function, including the release of exosomes. Hypoxia has already been reported to be a stimulus to increase secretion of exosomes by several groups [44–46]. It is also suggested that the protein and RNA content of exosomes can reflect the physiological state of the cell as well as when the cells are in stress condition [47, 48]. However, the initial stress insult that contributed to an alteration of the exosomal content in relation to the functional effects of the subsequent cargo transfer and their role in
cell-to-cell communication remains unclear. It is possible that exposure to other stressors such as adverse environmental hazards [49–51] will also increase secretion of exosomes and alter composition of the cargo.

The protein content of exosomes from ICAR cells cultured under the 1 % O2 contained unique proteins compared to the contents of the ICAR exosomes cultured at 8 % O2. Our proteomic analyses detected the presence of tetraspanin-6 (TSPAN6), disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) that are only unique to exosomes of ICAR cultured at 1 % O2. The proteins are involved in the biological processes for reproduction. Interestingly, to evaluate TSPAN6, belonging to the transmembrane 4 superfamily that mediate the regulation of signal transduction events, as well as the disintegrin-like metalloproteinase ADAM10 which participates in ectodomain shedding activity could provide great insights into their functional role and regulation that is important for reproduction.

Studies using immunohistochemistry of human placental explants [52] have demonstrated that ADAM10 expression is significantly increased in preeclamptic placentas compared with normal placentas. Up-regulation of ADAM10 could induce placental release of soluble vascular endothelial growth factor receptor-1 (sFlt-1) and this cascade is associated with endothelial dysfunction, suggesting the significant role of oxidative change in preeclamptic placentas. ADAM10 is also a shed- dase [53] that could induce CD46 shedding attributed to cell apoptotic processes [54], as well as mediate E-cadherin shedding affecting cellular adhesion and cell migration [55].

Mass spectrometry detection of pantetheinase (VNN1) in exosomes was unique to ICAR cultured at 8 % O2. VNN1 is an enzyme that hydrolyses pantetheine to form pantothentic acid (a precursor of coenzyme A) and the antioxidant cysteamine [56]. VNN1 could promote tissue inflammation through peroxisome proliferator-activated receptor gamma as well as modulate levels of glutathione [57]. It is proposed that VNN1 have innate immune functions and might contribute to tissue injury in endometritis [58, 59]. VNN1 was also reported being involved in proteolysis and can denature proteins by reducing disulfides [60], suggesting that it may have a role in regulating uterine receptivity for implantation and trophoblast invasion [61].

Mass spectrometry detected kininogen-2 (KNG2) in exosomes generated by ICAR cells cultured at either 1 or 8 % O2. KNG2 is a precursor protein to high molecular weight kininogen, low molecular weight kininogen and bradykinin, and the concentration were reported to fluctuate during ovulation, pregnancy, and parturition [62]. Studies also showed that the release of vasoactive bradykinins from high molecular weight kininogen and low molecular weight kininogen are responsible for micro-vascular permeability and vascular growth, which plays an essential role in utero-placental vasculature and angiogenesis, necessary for embryonic and fetal survival [63].

Conclusion
Our present findings show that ICAR cell function, release of exosomes and exosomal content can be altered when subjected to adverse stimuli. These findings should be expanded to include cells of endometrial epithelial origin, interactions between these cells (i.e. stromal—epithelial crosstalk) and in the presence of common pathophysiological factors associated with reduced fertility (e.g. infectious or inflammatory agents). The identification of unique proteins (by mass spectrometry) in exosomes of ICAR cultured at 1 % O2 compared to 8 % O2 suggests that the cells respond and release proteins encapsulated within the exosomes to signal the environment in which they live. It is hoped that identification of unique proteins in exosomes following stimulation by factors affecting the physiological condition of cows may lead to novel targets for manipulation to aid fertility. Moreover, inves- tigations into the release, uptake and content of exosomes may offer the opportunity to evaluate maternal-fetal crosstalk.

Abbreviations
ADAM10: Metalloproteinase domain-containing protein 10; DTT: Dithiothreitol; FDR: False discovery rate; ICAR: Intercaruncular stromal cell; KNG2: Kininogen-2; LDH: Lacrate dehydrogenase; MS/MS: Mass spectrometry/mass spectrometry; PANTHER: Protein analysis through evolutionary relationships; PBS: Phosphate buffered saline; PGF2α: Prostaglandin F2α; PVDF: Polyvinylidene fluoride; RIPA: Radioimmunoprecipitation assay buffer; RWD: Relative wound density; sFLT-1: Soluble vascular endothelial growth factor receptor-1; TSPAN6: Tetraspanin-6; VNN1: Pantetheinase

Acknowledgments
The authors acknowledge the assistance of Dr. Jamie Riches and Dr. Rachel Hancock of the Central Analytical Research Facility, Institute for Future Environments, Queensland University of Technology (QUT) for the electron microscope analyses. We also thank our colleagues at DairyNZ for their helpful insights. YQ Koh is supported by a student scholarship from a partnership fund (DRXC1302) between the New Zealand Ministry of Business, Innovation and Employment and New Zealand dairy farmers through DairyNZ Inc. CS holds a research fellowship at The University of Queensland Centre for Clinical Research, Brisbane, Australia. GER was in receipt of an NHMRC Principal Research Fellowship. These studies were funded in part by the Australian Research Council, Therapeutic Innovation Australian and National Collaborative Research Infrastructure Strategy.

Funding
Australian Research Council and a partnership fund (DRXC1302) between the New Zealand Ministry of Business, Innovation and Employment and New Zealand dairy farmers through DairyNZ Inc.
Availability of data and materials
The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

Authors’ contributions
YQK performed the study, collected and interpreted data performing statistical analysis and wrote the manuscript. YQK, SR, HNP and KV performed mass spectrometry analyses and reviewed the data generated. YQK, CS, HNP, GER and MDM were responsible for the study concept and participated in designing the study and interpreted data. CS, GER, HNP, MDM revised and approved the final version of manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

Received: 21 September 2016 Accepted: 25 October 2016
Published online: 09 November 2016

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