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Technical note

In silico analysis of gene expression in granulosa cells of preovulatory follicles in two species of bovines

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Abstract:

Buffaloes and cattle are two species of bovines with great similarity in their reproductive physiology, but at the same time with great difference in their reproductive parameters. The objective of this work is to compare gene expression in granulosa cells of preovulatory follicles of these two species, based on information available in the literature, existing transcriptome repositories and functional analysis using Ingenuity Pathway Analysis. Only two independent studies comparing buffalo and cattle in terms of gene expression in granulosa cells of preovulatory follicles were found. Expression data were analyzed independently and in combination. It was found that, between buffaloes and cattle, there is practically no correspondence between the processes evaluated, neither in the canonical pathways, nor in the upstream regulators, only some correspondence is found between the networks and physiological aspects of each process. It is concluded that each species has a different way of carrying out the same process and that each event should be studied according to the needs of the researchers.

Key words: Bovines, Granulosa, Transcriptomics, Follicle, In Silico.

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Buffaloes (*Bubalus bubalis*) and cattle (*Bos taurus*) are bovines closely related that are only differentiated by their mitochondrial DNA[1]. Buffaloes and cattle are seasonal polyestrous, with two or three follicular waves per estrous cycle[2]; however, under the same environmental and management conditions, their reproductive function is different, evidenced by the birth rate, expression of heat and response to reproductive biotechnologies. It has also been described that the buffalo has smaller ovaries[3], different follicular diameter at the time of deviation and ovulation[4], lower quality of oocytes and lower rate of embryo production compared to cattle. Li *et al*[5] found 40 loci (associated with 28 genes) that could be related to reproductive parameters such as age at first, second and third calving, days open, services per conception and calving interval[5,6]. The formation of a competent oocyte depends on follicular development, in one the formation of a preantral follicle with an oocyte capable of forming the individual and the second is the development of this follicle until ovulation, associated with all the endocrine changes that must happen in the female to achieve it[7,8].

The control of the follicular population depends on the function performed by the anti-Müllerian hormone (AMH), those who are going to ovulate must acquire receptors for follicle-stimulating hormone (FSH), finally some follicle gains receptors for LHr[8],which generates a decrease in the speed of growth that is maintained until the preovulatory peak of LH[9,10].

It has been reported that the buffalo oocyte is of poor quality when evaluated with the parameters normally used for cattle, and although the reasons are not known in detail, it has been proposed as a cause: an adverse follicular microenvironment, which has motivated researchers to study the proteins of the buffalo follicular fluid[11]. The existing communication between granulosa cells and the oocyte has been shown to play a critical role in producing good quality oocytes[12]. Nowadays, it is feasible the in-depth analysis of reproductive events in the female bovine. It is undeniable the progress achieved with microarrays and other systems used in transcriptomics, the data are deposited in repositories, which can be analyzed and allow the researcher to evaluate their premises. However, it is a heterogeneous base due to all the technical aspects and computational tools used for its analysis[13]. In a recent study, Khan *et al*[10] generated an interactive interface called GranulosaIMAGE, which provides information on gene expression profiles and their

isoforms in granulosa cells of cattle in different states of folliculogenesis (http://embbioinfo.fsaa.ulaval.ca/granulosaIMAGE/).

An important tool is Ingenuity Pathway Analysis (IPA), which is a database used for the analysis, integration and interpretation of data related to experiments based on information obtained by RNA-seq, principally, IPA allows, in a single analysis, identifying regulatory molecules, which facilitates the explanation of expression patterns, predicting the consequences of suggested control on cell biology and on the appearance of diseases[14]. The program makes the calculations based on algorithms and experimental data[15], in which the genes differentially expressed in the experiment are associated with those that have been most frequently reported on a particular function or metabolic pathway[16] and its control systems[17].

Developments in bioinformatics allow the study of biological phenomena in a complex way: in this case, the differences in the gene expression of buffalo and cattle follicles, in a more global way, which allow at least to consider all the possible genes that are expressed at a given time, showing the details of gene expression and the possible alternatives that nature has generated to carry out the same process. The objective of this work is to evaluate whether there are differences in the gene expression of granulosa cells of buffalo and cattle preovulatory follicles tending to seek explanations of the differential of the response to reproductive biotechnologies observed between the aforementioned species.

This work was carried out in two phases: the first, an exhaustive bibliographic search was carried out in the databases of PUBMED and Google Scholar, focusing on information corresponding to the analysis of gene expression between buffaloes and cows and whose files were deposited in GEO (Gene Expression Omnibus) until March 2018, the second, the analysis of the reports found was carried out. The inclusion criterion was: experiments where there was comparison of data; or if there was no comparison, that the data were obtained in the same way and processed in a similar way.

Two studies of transcriptomes of preovulatory follicle were found[18,19]. In the case of cows, RNA was extracted with RNeasy mini kit (Qiagen) and in buffalo with TRIZOL followed by purification with RNeasy mini kit, cDNA was obtained in both cases, and they were hybridized with an Affimetrix Chip (Affymetrix Gene - Chip Bovine Genome Arrays), which contained 24,128 probes, representing 23,000 transcripts and their variants, including 19,000 unigene clusters. Those genes with a change in expression ≥2 with a false discovery rate (FDR < 0.05) were considered as expressed differently, subsequently, a semi-quantitative RT-PCR was performed for the validation of the results with the following genes: LH receptor (LHR), progesterone receptor (PR) and cyclooxygenase COX-2 in buffaloes, and two cytochrome P450 genes related to estrogen production, Cytochrome P450 CPY19A1, CPY17A1, 18s, for cattle.

Subsequently, the raw data from each microarray were corrected by background noise removal and normalization (loess) using the FlexArray 1.6.1 software, and all data were exported to excel (Microsoft Office) files to be functionally analyzed using Ingenuity® Pathway Analysis (IPA). UMD *Bos taurus* 3.1.1 was used as the comparison genome for both cases since the buffalo genome is not yet fully sequenced. It was obtained from a list of differentially expressed genes of the preovulatory follicles of each species and this difference was quantified, they were grouped according to their biological function and potential upstream regulators. The information was processed individually and compared between the two species. The program works by assigning a probabilistic value to the association between over- or under-expressed genes and major biological functions. For the analysis of upstream regulators, IPA® compares the results of the experiment with its own database of the known effects of genes and molecules on expression. Two values are calculated from each regulator, an overlap value (overlap p) and an activation value (Z score), which corresponds to a calculated numerical average of the known effects of the molecule or gene (up or down regulation) and their respective targets. A Z value is considered significant when it has a value greater than 2 (Z-score > 2) for activation and (Z-score < 2) for inhibition, intermediate values are considered not to be attributable to the experimentation[14].

Twenty-one reports on gene expression in bovine granulosa cells in which there was at least one database deposited were found, and of these, only one had information on buffaloes. There were no reports on the comparison of differential gene expression between buffaloes and cows. Finally, the requirements were met by two reports: "Transcriptome profiling of granulosa cells of bovine ovarian follicles during growth from small to large antral sizes", access number GSE39589 with four repetitions[19], and "Buffalo Gene expression profiling of preovulatory follicle in the buffalo cow: effects of increased IGF-I concentration on periovulatory events", access number GSE11312, with three repetitions[18].

In the initial works, it is reported that, in buffaloes, there are 110 differentially expressed genes that belong to 14 metabolic pathways according to the Gene Ontology database, for cows, 446 genes belonging to 10 metabolic pathways were found.

The IPA analysis shows that the most important canonical metabolic pathways associated with the expression pattern observed in the buffalo were: Protein ubiquitination, mitochondrial dysfunction, oxidative phosphorylation and signaling associated with the estrogen receptor and sirtuins, in cows, the most important canonical pathways are the synthesis of triacylglycerides, signal translator and activator of transcription 3 (STAT3), phagosome maturation, Janus Kinase 2 (JAK 2) Protein in the signaling of cytokine-like hormones and that of oncostatin (Table 1).

Table 1: Main metabolic pathways in the buffalo and cow follicle

Pathway	p-value	Overlap (%)	Molecules
Buffalo follicle			
Protein ubiquitination	1.11 E -11	19.70	53/269
Mitochondrial dysfunction	2.41 E-11	22.30	42/188
Oxidative phosphorylation	7.80 E-10	25.20	30/119
Signals of the estrogen receptor	9.46 E -10	23.90	32/134
Sirtuin signaling	3.46 E -08	16.00	52/325
Cow follicle			
Triacylglycerol biosynthesis	1.41 E-03	6.90	4/58
STAT3 pathway	1.83 E -03	4.80	5/104
Phagosome maturation	1.97 E-03	3.90	6/155
Role of JAK2 signaling*	2.84 E -03	8.80	.3/34
Oncostatin M signaling	4.53 E-03	7.50	.3/40

*cytokine-like hormone.

The IPA analysis showed that the genes or molecules of the following pathways: HNF4A, RICTOR, EIF4E, 1-2-dithiole-3-thione, STI926 were activated in buffaloes, without being any characterized in buffaloes, and for cows, they were: transforming factor beta 1 (TGFB1), Estrogen receptor type B2 (ERBB2), dexamethasone, beta-estradiol, D-glucose (Table 2).

Table 2: Main upstream regulators

	p-value
Buffaloes	
HNF4A	2.70 E-27
RICTOR	2.15 E -17
EIF4E	1.38 E -15
1,2-dithiole-3-thione	4.9 E-15
ST1926	5.64 E-15
Cows	
TGFB1	7.64 E-09
ERBB2	1.2 E -08
Dexamethasone	1.3 E E-07
beta-estradiol	5.21 E-07
D-glucose	2.10 E -06

The most important functional networks for buffaloes were: 1) molecular transport, RNA trafficking and post-translational modifications, 2) cell signaling, post-translational modifications and protein synthesis, 3) cell assembly and organization, developmental and hereditary disorders, 4) post-transcriptional RNA modifications, protein synthesis and gene

expression, cell maintenance and function, post-translational modifications, and protein folding. For cows 1) cell organization and assembly, developmental disorders and neurological diseases, 2) lipid metabolism, small molecule biochemistry, intracellular signaling and interaction, 3) cell death and survival, interaction between them and cancer, 4) molecular transport, cell signaling and vitamin and mineral metabolism (Table 3).

Table 3: Most important functional networks

Name of networks and their functions	**Score**
Buffaloes	
Molecular transport, RNA trafficking and post-translational modifications	153
Cell signaling, post-translational modifications and protein synthesis	146
Cell assembly and organization, developmental and hereditary disorders	141
Post-transcriptional RNA modifications, protein synthesis and gene expression	139
Cell maintenance and function, post-translational modifications and protein folding	123
Cows	
Name of networks and their functions	Score
Cell organization and assembly, developmental disorders and neurological diseases	163
Lipid metabolism, small molecule biochemistry, intracellular signaling and interaction	79
Cell death and survival, interaction between them and cancer	73
Molecular transport, cell signaling and vitamin and mineral metabolism	11

In this work, gene expression in preovulatory follicles between two closely related species was compared, the way to do it is a novel approach to have more knowledge. At first glance, there is no strict relationship between the concepts enunciated in the introduction and the results of the experiment. This fact can be considered to be discussed because before doing the analysis, the thought of the researchers was focused on the existing knowledge about endocrine control and follicle development in the species and the results show information associated with aspects related almost exclusively to cell and molecular biology.

It is important to highlight the difficulty in finding comparable information, and given the biological approach of the writing, it is decided to avoid the technical discussion on the way of obtaining the data and its analysis, which is so frequent in this type of publications. In a previous work of the group[20], where the information reported in the two articles evaluated in this study is described, it is shown that in the preovulatory follicles of cows and buffaloes, there is only coincidence in the expression of three genes (20 %): tissue plasminogen activator (PLAT), steroidogenic acute regulator, (STAR), coagulation factor II receptor-like I (F2RL-1), with differences in expression levels (relative expression) 9.7 *vs* 17.5, 8.74 *vs*

3.4, and 7.7 *vs* 5.3 times for PLAT, STAR and F2RL-1 respectively, showing the differences between species. Additionally, it has not been reported that any of the genes found have to do directly with ovulation, follicular development or that they are markers of the same biological processes.

PLAT is a secreted serine protease that converts the proenzyme plasminogen into plasmin, a fibrinolytic protein. An increase in its function causes hyperfibrinolysis, which is evidenced by excessive bleeding, and a decrease in its function causes hypofibrinolysis, which is evidenced by thrombosis and embolism, it has been reported overexpressed in granulosa cells of ovulatory follicles and it has been associated with follicular rupture[21].

STAR has an important role in the acute regulation of steroid hormone synthesis, allows the breakdown of cholesterol to pregnenolone to facilitate its transport from the outer membrane to the inner one in the mitochondria. In buffaloes, it has been reported that the expression is increased (up regulated) in granulosa cells and follicular wall after the bovine growth hormone treatment[18], additionally, a synergism between insulin-like growth factor 1 and gonadotropins to increase the STAR expression[22].

F2RL-1 is a protein that belongs to the family of those that are associated with the type I receptor of G proteins, also has a function on the inflammatory response, in innate and adaptive immunity[23]. Signaling through this gene (F2RL-1) mediates the *in vitro* cytogenesis of endothelial cells and promotes vasodilation and microvascular permeability, fundamental steps of angiogenesis. Overexpression of the F2RL-1 gene has been observed, partly explaining the growth changes of the blood vessels of the theca cells, and the invasion of the mural granulosa cells after follicular rupture[24].

Although there is a low correspondence between the two species, common genes are associated with ovulation mechanisms, genes associated with the way the cell performs its function. No differentially expressed genes are observed associated with the phenomena that direct the process, such as gonadotropins or sex steroids. Consequently, a new way of viewing and analyzing the data obtained must be found since what was found is rather the description of how a phenomenon is executed, which, in this case, would be the proteolytic factors for the rupture of the follicle, the changes in the vasculature for the remodeling of the organ and the accumulation of estrogens to generate the LH peak. For some authors, ovulation has been associated with inflammation[25], it could then be assumed that the two species do the same, with a group of general master molecules and another of effectors that can be very specific and mark the differences observed.

Protein ubiquitination is the most important pathway for the degradation of half-life regulatory proteins. Hatzirodos *et al*[20] found that this pathway is involved in the transition

from a small to large follicle. Other researchers found it as a regulator of androgen receptors in humans[26].

Mitochondria are the main oxygen consumers of the cell; the effect of the proper functioning of the pathway on oocyte competition for its role in the formation of reactive oxygen species has been reported[27]. In women, mitochondrial transfer has been proposed as an alternative for the treatment of oocytes from patients with diseases that include alterations in the mitochondria[28], also in obese humans, that mitochondrial dysfunction is associated with alterations in fertility. In buffaloes, it has been reported that supplementation of maturation media to produce embryos *in vitro*, with cystine with or without cysteamine significantly increases the proportion of oocytes exhibiting normal fertilization, cleavage and blastocyst production[29].

Oxidative phosphorylation is the process by which the cell produces ATP. Li *et al*[6] reported, in buffaloes, that oxidative phosphorylation is important for the early stages of follicular development. Its role in the ovulatory process given the hypoxic nature of the process has also been reported. Evidence that the follicle needs to produce more molecules for the hypoxic response in the preovulatory antral follicles has been shown [5].

Sirtuins belong to class III of deacetylase enzymes that use NAD+ as a substrate to perform their function, it has been reported that there are 7 in mammals with multiple functions (SIRT 1-7), they have function as regulators of transcription in aging, metabolism, cancer, inflammation, DNA repair and cellular response to stress[30].

SIRT1, SIRT2 and SIRT3 have been reported to have protective function for oocyte aging after ovulation, in addition, SIRT3 has a role in the detection of reactive oxygen species (ROS) in folliculogenesis and luteinization processes of granulosa cells[31]. Pacella-Ince *et al* have described that low mRNA levels of mitochondrial SIRT 5 are associated with reduced ovarian reserve and ovarian aging in humans[32].

Most important metabolic pathways in cows:

Triacylglycerol biosynthesis. The effect of lipid droplets on oocyte quality and function is widely documented in the literature[33]. Today, they are considered as a separate organelle with their own associated enzymes. In humans, the lipid profile of granulosa cells has been successfully associated in gestation, in cow oocytes, it has been associated with quality, and in humans with effect on maturation[34].

Phagosome maturation is a process in which some particles that are internalized move on acidified structures, which are integrated into a mechanism of apoptosis. This process necessarily involves maturation, which can be seen by the sequential fusion of the different

stages of lysosomes that end the formation of a phagolysosome. There is very little information about the role of this pathway in follicle formation or oocyte quality; it has been suggested as an alternative pathway for the control of oocyte death and that it can be activated during follicle formation for autophagy processes. Alteration of some genes encoding proteins for autophagy cause a large loss of oocytes, suggesting a role of autophagy in regulating their survival[35].

Janus kinases are a family of 4 tyrosine kinases. These play an important role in cell growth, survival and differentiation; they are widely expressed in the preovulatory follicle and their expression is inhibited by intrafollicular injection, it has also been reported that in granulosa cells, IL-6 promotes FSH-induced VEGF expression by the JAK/STAT3 pathway[36].

Cytokines are the main intercellular mediators, these activate in the ovary immune and non-immune cells that facilitate the constant reorganization of the ovarian stroma through proteolytic pathways, facilitating modifications of the basement membrane of granulosa cells, and endothelial vascular invasion. Martins *et al* reported that receptors for oncostatin M are regulated in granulosa cells during follicular atresia, ovulation, and luteolysis, and that oncostatin protein from other cells regulate the function of cell viability genes in granulosa and luteal cells[37]. Many molecules involved in the follicular development that affect signals for ovulation trigger the expression of cytokines such as TNFa, interleukin 7, and CDKN1A[38].

When the comparison of gene expression between the two species is made and the 10 activated canonical pathways with the highest P value are analyzed, only a few are shared with the individual analyses, in the case of buffalo, 5 of the 10, while for cows only 1 (Table 4).

Table 4: Comparison of the main metabolic pathways in the two species

Metabolic pathways	p-value	Relationship	Score- z
Sirtuins signaling pathway	5.02E+00	1.63E-01	2.191
Role of CHK proteins. Proteins in cell cycle control	4.74E+00	2.78E-01	1.897
Oxidative phosphorylation	4.61E+00	2.19E-01	-4.583
De novo biosynthesis of pyridine ribonucleotides	4.38E+00	2.89E-01	-1.941
Mitotic role of the Polo-like kinases	4.07E+00	2.46E-01	-1.667
Interconversion of pyrimidine ribonucleotides	3.93E+00	2.79E-01	-1.732
Androgen signaling	3.85E+00	1.83E-01	0.378
3-phosphoinositide degradation	3.76E+00	1.74E-01	-1.569
Regulation of eIF4 and p70S6K signals	3.67E+00	1.72E-01	0.632
Biosynthesis of D-myo-inositol (1,4,5,6)-tetrakisphosphate	3.65E+00	1.78E-01	-1.225

Upstream regulators in buffaloes:

Hepatocyte nuclear factor 4A (HNF4A) is involved in gluconeogenesis and lipid metabolism. This factor has been reported by Khan *et al*[11] as an upstream regulator in cow granulosa cells after being stimulated with FSH. RIPTOR is a gene that produces a molecule that accompanies the target of rapamycin. In human cells, it has been reported that an mTOR-associated kinase is necessary for Ser^{473} to be phosphorylated, inhibiting the AkT/PKB metabolic pathway that is involved in apoptosis and oocyte maturation.

The translation initiation factor, EIF4E, produces a protein that helps the start of translation by recruiting ribosomes at the 5'-end of the subunit, limiting the start of translation. Its function is reported to be increased in the transition from primordial follicle to primary follicle[39], in the oocyte for the initiation of mRNA translation associated with the continuation of meiosis, and in the inhibition of the TORC1 function for the consumption of amino acids.

IPA also reports on the role of some regulatory molecules of gene function, evaluates genes associated with function control such as 1-2-dithiole-3-thione, which has effect on antioxidant enzymes, and ST1926, which is a retinoic acid-like molecule with effects on

growth and differentiation[40]. There are no reports of the action of these molecules on the function of granulosa cells.

Upstream regulators in cows:

The transforming factor beta-1 belongs to the superfamily of proteins of the transforming factors beta, they have been described in many regulatory activities in the ovary, follicular development and ovulation in different species. Landry *et al* report that it has a role in the production of a competent oocyte and in the induction of atresia in the case of persistent follicles[10].

ERBB2 encodes a tyrosine kinase that belongs to the family of epidermal growth receptors, amplification or overexpression of this gene has been associated with breast and ovarian tumors[30] and follicular development[19], in the regulation of the expression of the TP53 tumor suppression gene during the proliferation of granulosa cells under the stimulation of FSH and after the LH peak[41].

Estrogens, Dexamethasone and D-Glucose are molecules reported with effects on follicular development in cattle[42]. Estrone and estradiol are steroid hormones synthesized in the ovary, critical for reproductive function, the main enzyme of this pathway is the aromatase CPY19A1[43].

At this point, it is easily understandable that, despite being bovines, each species has its different way of performing the phenomenon, and that is one of the important results of this work, the evidence shows that each species develops the follicle in a different way. Since it is observed that each species ovulates with different events, the objective of explaining the reproductive differences in reproductive behavior between these two species cannot be met.

The high correspondence of the result obtained when analyzing the most frequent biological networks or functions in the studied event between the two species analyzed, it is shown that both are having the same biological event: growth of a structure within the organism with high metabolic production, cell proliferation, production of signals for all the events that will happen, whose consequence is ovulation. The comparison between networks shows how they do the same with a different number of molecules, buffaloes involve more molecules than cows, which could give them an advantage for ovulation, having more options of cellular pathways available. In the comparative analysis between species, buffaloes do not only share the pathway of lipid metabolism with cows, suggesting that there should be differences at this level in follicular fluid or oocytes, with no reports to date in the literature.

Only a few publications in reproduction have tried to make comparisons between buffaloes and cows with a global approach to the problem, it is interesting to see how the evidence of

the differences is increasing. Reports on differences in specific genes in specific events, such as signaling for meiosis restart[44], or the role of transforming factors on follicular development[45], can easily be found. Much information is known about the physiology of granulosa cells and their role in follicular development in cows, but it is scarce in buffaloes and much less its comparison. There is only one article in the study of the transcriptomics of buffalo granulosa cells, using RNA-seq and slaughter plant material, finding differential expression in 595 genes when comparing the initial states with the end states of follicular development[5].

The comparison between species is a novel approach in the area of reproductive biology, studying the same phenomenon on equal conditions between buffaloes and cows shows how each species has developed its own way of carrying out its processes, that the regulation of the same have different pathways between *Bos indicus* and *Bubalus bubalis*, so the phenomena must be studied in a particular way for each species and that the extrapolation of the information obtained between species should be avoided and be the basis for the analysis of the complexity of the phenomena studied, with the obligation to see them in a different, non-reductionist way.

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