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Brief Communication

Gene expression analysis of bovine granulosa cells from growing follicles

Jesus Berdugo-Gutiérrez[1*]; Ariel Tarazona-Morales[2]; Carlos Muskus-López[3]; Julían Echeverry-Zuluaga[1]; Albeiro López-Herrera[1].

[1]Universidad Nacional de Colombia, Facultad de Ciencias Agrarias. BIOGEM. BIOGENESIS. Medellín, Colombia.
[2]Universidad Nacional de Colombia. Facultad de Ciencias Agrarias. Medellín, Colombia.
[3]Universidad de Antioquia. Unidad Biología Molecular y computacional PECET. Medellín, Colombia.
*Correspondencia: jaberdugog@unal.edu.co

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ABSTRACT

Objetive. This work compares granulosa cell gene expression using RNA analysis from pre-ovulatory follicles from two different bovine species (buffaloes and cattle). **Materials and methods.** The RNA was obtained from granulosa cells from ovaries of 10 buffaloes and cattle obtained at the local slaughterhouse, was sequenced with Novaseq, and the differential expression was analyzed using EdgeR in Bioconductor, and the function was assigned according to gene ontology terms. **Results.** Differential gene expression analyzes shown significant differences between species, but the most important feature is the low participation of genes associated with the reproductive process of follicular development, highlighting the importance of paracrine control of the ovary. It was found that between buffaloes and cattle, there is practically no correspondence in the gene expression of the physiological states evaluated; 6137 genes show differential expression between the two species**. Conclusions.** Each species has its way of performing the same process. The differences in the expression of the genes associated with oxidative phosphorylation are evident, and new ways to look at the presented results are required to understand the biological significance of the findings.

Keywords: Buffaloes; granulosa cell; ovarian follicle; transcriptome; RNA-Seq (*Fuente: MeSH*).

RESUMEN

Objetivo. Comparar la expresión génica en células de granulosa de folículos en crecimiento en dos diferentes especies de bovinos (búfalos y vacas). **Materiales y métodos.** El RNA obtenido de las células de granulosa de 10 vacas y búfalas vacías fue secuenciado con Novaseq y se analizó la expresión génica diferencial usando EdgeR en Bioconductor y su función de acuerdo con los términos de ontología génica **Resultados.** El análisis de expresión diferencial mostró grandes diferencias entre las especies, fundamentalmente, la poca participación de los genes asociados a los fenómenos reproductivos del desarrollo folicular, poniendo de manifiesto la importancia del control paracrino del ovario. Se encontró que, entre búfalos y vacunos, no hay correspondencia en la expresión génica de los estados fisiológicos evaluados y aunque se pudieron identificar 6137 genes que tienen expresión diferencial entre las dos especies, no se encontró significancia en genes asociados directamente sobre el desarrollo folicular. **Conclusiones.** Cada especie tiene su forma de realizar el mismo proceso, aunque son evidentes las diferencias en la expresión de genes asociados a la fosforilación oxidativa. Se requieren nuevas formas de analizar los resultados para poder entender el significado biológico de los hallazgos.

Palabras clave: Búfalos; célula de granulosa; folículo ovárico; transcriptoma; RNA-Seq (*Fuente: MeSH*).

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INTRODUCTION

Many productive systems were developed to produce protein from the animal origin using phylogenetically close related animals. Cattle and buffaloes are mammals and ruminants, thanks to this, it has been thought that many of the aspects of their physiological responses could be extrapolated from one to each another, but the evidence shows that this is not so true.

Buffaloes (B*ubalus bubalis*, 2n = 50) and cattle (*Bos indicus*, 2n = 60) are bovines, differentiated only by their mitochondrial DNA; based on studies of the differences in amplification of polymorphic fragments (AFLP), three tribes have been described: buffalo (African and water), cattle (bullfighting and zebu) and bison (American and European) (1).

Buffaloes and cattle have a similar reproductive pattern: monoovular, polyestric, seasonal, with two or three follicular waves per cycle. However, buffalo have been reported to have smaller ovaries, different follicular diameter at deviation and ovulation, lower oocyte quality score, lower *in vitro* embryo production rates, a different expression of heat symptoms, and response to reproductive biotechnologies (2). When comparing some reproductive parameters such as the birth rate, expression of heat symptoms, and response to reproductive biotechnologies, in animals maintained in the same environment and management, the two species show important differences (3), paradoxically natural reproduction is better in buffaloes, and better response is seen in cattle in artificial reproduction.

Ovulation is an adaptive mechanism developed by nature to ensure that a competent oocyte, capable of forming an individual, is delivered within an endocrinologically suitable genital tract to allow the implantation of an embryo and develop to term. To date, the protocols for the manipulation of ovulation are based on the induction of a new follicular wave at a given moment of the estrous cycle that needs to produce a competent oocyte. (4). To create a competent oocyte, communication between the granulosa cells and the oocyte is essential; consequently, it would be expected that the genes responsible for these actions would have a determining role in reproductive parameters. Folliculogenesis requires the coordinated action of numerous intrinsic and extrinsic factors, determined by the granulosa and the oocyte (5), which allow constructing an idea of the follicular functioning based on the knowledge of gene expression. Due to a large amount of information on these interactions, Khan et al. (6) generated an interactive interface called GranulosaIMAGE, which reports on the expression profiles in the granulosa cells of cattle at different stages of folliculogenesis (HTTP: // emb bioinfo.fsaa. ulaval.ca / granulosaIMAGE /).

In mammals, follicle recruitment occurs by escaping the growth suppression of antimullerian hormone (AMH) by acquiring receptors for follicle-stimulating hormone (rFSH). Follicle growth is dependent on the action of the FSH until the deviation (selection of the dominant follicle), which occurs in cattle when they reach 8.5 mm and in buffaloes at 7.5 mm. After, the follicle produces luteinizing hormone receptors (LHr), which generates a decrease in the growth rate that is maintained until the pre-ovulatory peak of LH. (7)

The use of molecular biology tools associated with bioinformatics developments allows progress in understanding biological processes. Microarrays and next-generation sequencing have allowed advances in gene expression analysis; in a single run, the expression of a large part, if not all, of the genome can be evaluated. Additionally, the information on a phenomenon can often be consulted in public databases that could be used for analysis, considering that it is heterogeneous due to the technical aspects and the computational tools used for its analysis (8).

Li et al (9), using single nucleotide polymorphisms (SNPs), studied in granulosa cells from different sizes the genetic bases of the reproductive behavior of 462 Mediterranean buffaloes and found 25 SNPs distributed in 13 genes associated with reproductive parameters. Of them, 11 were expressed in follicles of all sizes; there were only differences in the expression of the NDUFS2 gene between follicles larger and smaller than 8mm. In another report, the same authors (9), using DNA from peripheral blood lymphocytes, evaluated reproductive parameters and SNPs and found 40 loci, within 28 genes associated with age at first calving, second and third birth, days open, services per conception, the interval between parturitions, it was possible to confirm that 25 of the 28 genes are also expressed in granulosa cells and that IGFBP7 gene is present throughout the follicular development (10).

There has been a growing interest in the search for explanations for the differences between species, using comparative evaluations to develop knowledge and new concepts in reproductive biology to offer buffalo breeders alternatives for the improvement of their herds. The present work aims to compare the gene expression results in growing buffalo and cattle follicles by RNAseq analysis.

MATERIALS AND METHODS

Study site and sample collection: This work was performed at the Biotechnology Laboratory of the National University of Colombia-Medellín. Ovaries from non-pregnant ten cattle (*Bos indicus*) and ten buffaloes (*Bubalus bubalis*) were obtained from the local slaughterhouse were taken to the laboratory in saline solution at 37ºC, within two hours after slaughter. All follicles smaller than 7 mm from each ovary were aspirated, the follicular fluid obtained was transferred to a Petri dish. With the aid of a stereoscope, the granulosa cells were identified and put into vials with 100ul of Phosphate buffered saline PBS plus 400ul of RNA later® (Applied Biosystems), frozen at -20°C until RNA extraction. The purity of RNA was evaluated by determining the absorbance A260/280 and A260/230. The RNS integrity number (RIN) was calculated using the Bioanalyzer 2100 Bioanalyzer (Agilent Technologies). Samples with RIN greater than five and a ratio A260/280> 1.8 were used for analyses.

RNA extraction, cDNA synthesis and sequencing: RNA were extracted using a commercial kit base don silica gel membranes and columns, following the manufacturer instructions (RNA easy Mini Kit®, Qiagen), after extraction to remove possible DNA contamination, DNAase RNAase- free were used (Takara, China). Four 4 µg of purified RNA were sent to Macrogen (Corea). They constructed the cDNA library using TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA). The quality of the library was performed using the Bioanalyzer 2100 Bioanalyzer (Agilent Technologies). The fragments obtained were amplified and sequenced using the NovaSeq 6000 S4 Reagent Kit, where readings with paired ends of 100bp were obtained.

Assembly and Annotation. The quality of the reads was evaluated using FastQC Version 0.11.3, and the quality control was performed removing the adapters; only reads with a value of Q>20 over 30% and no more than 5% of unidentified nucleotides were included in the analysis.

To map the cDNA fragments, software HISAT2 and BOWTIE and For comparison, GCA_000247795.2 bovine genome was used (11). After alignment using StringTie, the intensity of expresión was calculated using FPKM (Fragments Per Kilobase of transcript per Million de lectures mapped) as the value, based on the numbers of reads and their normalized value.

For the expression analysis, those genes with 0 lectures were excluded. The data were transformed and normalized using the EdgeR library from Bioconductor; the criteria to be considered a differentially expressed gene was false Discovery rate (FDR≤0.05) and $\log_2$Ratio ≥1 (12). The cluster of expressed genes was performed after normalization using the euclidian linkage method (12). The enrichment analysis was performed using the available information in Gene Ontology using the g: Profiler tool (https: bit.cs.ut.ee/gprofiler/).

RESULTS

The amount of RNA obtained was a limitation in this work. After processing to get libraries, only a cattle and a buffalo sample could be compared. In table 1, the information related to the material used for analysis could be seen; 65.87% and 93.46% of the buffalo and cattle reads were mapped in the reference genome. For the analysis, 26,229 genes were evaluated, of which only 15062 could be compared, and of these 6137 fulfilled the statistical parameters of differential expression (fold change> 2; p<0.05), being 49.7% overexpressed and 51.3% under-expressed. After the comparison between cattle and buffalo, the top 10 overexpressed genes differentially expressed were: LRRC8D (Leucine 8 repeasts D subunit), KYRU (Kiruneninase), MTPN (Miotrofine), SERPIN A11 (Serpin family variant No 11), SYN2 (Sinapsine II), FAM219A (Family with sequence similarity 219 member A), SFRP2 (Secreted Frizzeled Protein 2), NTS (Neurotensine), MSMB (microseminoprotein beta) LTB4R (Leucotrien Receptor B4). The top 10 underexpressed genes were COX3, COX2, COX1 (Cytochrome B Oxidase 1, 2, 3) y ND3, ND4, ND5, ND2 (NADH deshidrogenase 3,4,5), CYTB (Cyitochrome oxidase B), LUZP6 (Leucine zipper 6) y ACCSL (1-aminocyclopropane-1-carboxilase synthase homolog like).

Table 1. General parameters of the simples used for the analysis.

Parámeter	Buffalo	Cattle
Reads mapped	31.292.230 (65.87%)	59.183.028 (93.46%)
Total read bases	4.893.015.296 bp	6.539.398.924 bp
Reads	48.445.696	64.746.524
G/C %	51.83	50.30
Q30	94.60	94.77
Reads after adapter removal	47.508.526 (Q30 - 95.47%)	63.326.588 (Q30 - 95.70%)
Total genes	33505	26229
Excluded genes	17333	11167
Included genes	16182	15062
Genes used for comparison	6137	
Comparison parameters	|fc|>=2 & p<0.05	|fc|>=2 & p<0.05

From the gene expression análysis, there are differences in clustering between buffaloes and cattle, and one example is the genes associated with the respiration chain. (Figure 1).

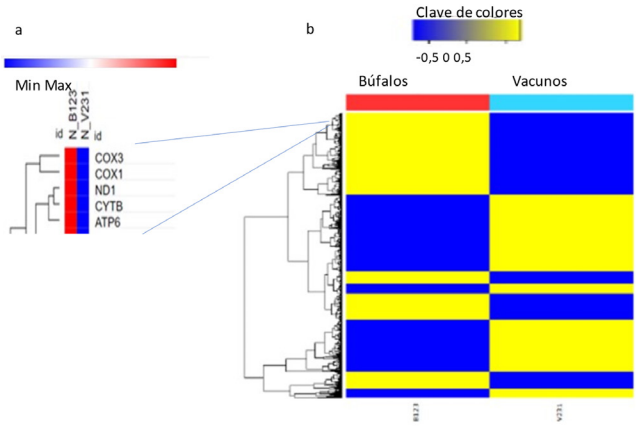

Figura 1. Heat map of differential expression between cattle and buffaloes grouped by clusters a. An example of the differences showing electron transport chain b Heat map of differential gene expression.

Evaluating the gene ontology terms show that the five more important biological processes were: cellular component organization and biogenesis, cellular component organization, positive regulation of the biological process, developmental process, anatomical structure development. The five principal terms associated with a function of molecules were: binding, binding to proteins, catalytic activity binding to ions to metals y. The final aspect of ontology terms was: intracellular, cell, and part of them.

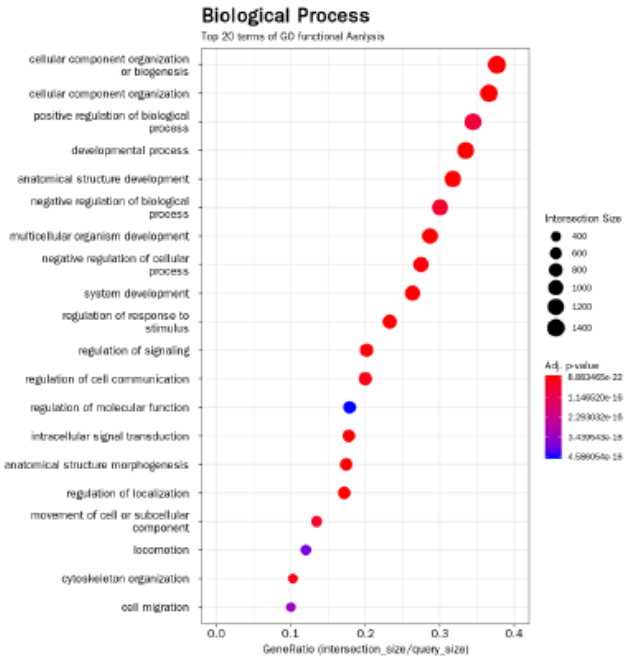

Figure 2. Grouping on ontology terms based on Biological terms.

DISCUSSION

This is the first report in the literature about the differential gene expression of the granulosa cells between river buffalo (*Bubalus bubalis*) and cattle (*Bos indicus*) since most of the studies are performed in swamp buffalo (9). Due to the small number of samples that met the quality requirements for sequencing, the authors have been decided to send this work as a case report. Additionally, from a technical point, it has been reported that the low concentrations of RNA and low quality after extraction for sequencing were obtained as limitations for this type of experiment (8).

This report has focused on comparing genes at the same stage of follicular development between the two mentioned species; it is observed that buffaloes and cattle have different expression patterns. Looking for comparisons in the literature, these results were compared with GSE39589 (12), GSE11312 (13), transcriptomic data from pre-ovulatory follicles from cattle and buffaloes, respectively, finding differences in

gene expression, as the results obtained in this work.

Comparison of the differentially expressed genes shows that none of the overexpressed genes have a known function on folliculogenesis or oocyte maturation; it is important to mention that under-expressed genes are all related to the respiratory chain. It will be necessary to evaluate if this difference could explain the observation performed by Marin et al. on the increase in the production of free radicals in buffalo *in vitro* embryo cultures and the need to add antioxidants to increase *in vitro* embryo production rates (14).

Evaluating some genes associated with follicular development and gene expresión, it has been obtained that AMH, INHA INHBA, PTEN, and VEGFD VNN1 were in buffaloes underexpressed and IGF2R was overexpressed. Other genes such as AMHR, FSH, p53, CDK1, CYP51A1, CYP19A1, FOXO 1, LHR, TNFA don´t show differential gene expression between two species.

Li et al. evaluated the buffalo genome looking for some markers and genes that could be associated with reproductive parameters, identifying 13 candidate genes (10) that were expressed in small follicles, comparing these results with the obtained here only NDUFS2 Y ARID4B were differentially expressed the other: PRDM5, GDF9, COL23A1, SCG5, PELI2, ABCC4, ACCS, TPCN1, TBCB, CDH10, LY86 don't have changes in gene expression between two species, additionally again follicular development genes don't have differential expression.

The evidence then shows that the search for explanations for the differences observed in reproductive parameters is not associated with the expression of the genes directly involved in the development of the follicle. Some genes are associated with inflammation (5). It is very important to keep in mind that the differential expression of a few genes may have a function in the context of the entire transcriptome of the cell that could be more important than genes associated with the execution of functions.

The gene ontology analysis shows how the same processes happening to the granulosa cells in both species have many molecules for binding, something common in the cells studied since the phenomena of follicular development are governed by the FSH and LH gonadotropins produced. In the pituitary, so what gonadotropins ultimately do is activate pathways such as MAP Kinase to facilitate the proliferation of cells and the expansion of cumulus (15). Other works in buffalo antral follicles show that the signaling pathways for the functioning of ribosomes and oxidative phosphorylation are enriched (16). Additionally, the expression of molecules of the TGFβ family produced by the oocyte affects granulosa cells gene expression, evaluated in this experiment, did not have any differences.

It should be taken into account that beyond the presence or absence of expression or specific genes, the samples used in this experiment correspond to follicles that are growing follicles before follicular deviation that they have an oocyte that has reached its maximum size (17).

The finding that the expression of genes associated with binding is in accordance with the physiological nature of the follicles. The evaluated follicle size needs proliferative tissue/cells for its development. Consequently, a large amount of messenger RNA for signals is required (18) to fulfill its function: immediate ovulation and indirectly a competent oocyte to form an embryo. Other papers associated with the comparison of differential gene expression in follicles report 110 differentially expressed genes in buffaloes belonging to 14 metabolic pathways and that 446 genes belonging to 10 metabolic pathways were found in cows (14).

Only a few publications on reproduction have tried to compare buffaloes and cows with a global approach to the problem. Most publications tend to confirm specific results of the differences reported here and make general postulates of the studied biological process. It is straightforward to find reports on the differences in specific genes, in particular events, such as signaling for the restart of meiosis (5), or the role of transforming factors on follicular development. Much information about the physiology of granulosa cells and their role in follicular development in cows is known. Still, it is scarce in buffalo and much less common in its comparison. To date, there is only one paper related to the study of the transcriptomics of buffalo granulosa cells; it uses RNAseq from ovaries recovered in a slaughterhouse: finding differential expression in 595 genes comparing initial stages with final stages of follicular development. (10). The comparison between physiological events shows that the animals do the same with different molecules. Buffaloes involve more molecules than cows which could give them an advantage

for ovulation by having more options of cellular routes available

It should not be forgotten that less than 1% of the annotated genes have an expression restricted to one type of tissue (19); therefore, derived from sequencing studies, the main finding will always be the baseline expression of all genes for life maintenance, it will be inevitable to obtain thousand of genes. But it is essential to understand that the analysis will depend fundamentally on the context of the experiment and the researcher's objectives. They will have to be analyzed with great care before carrying out an experiment that generates thousands of data, which could confuse rather than help the researcher.

The high correspondence between the results obtained here associated with the analysis of biological networks or functions shows that the two species have 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, the consequence of which is ovulation.

In conclusion, with the limitations associated with the size of the sample studied, the findings obtained here show that there is not the same pattern of gene expression to do the same biological phenomenon. Despite being bovines, buffaloes, and cows, each one has its specific way for the development of follicles. It could be assumed that despite using the same signals, they do not involve the same molecules.

The comparison between species is something new, especially in reproductive biology; it has been shown that studying the same phenomenon under the same conditions between two closely related species: cows, shows how each species has developed its own way of carrying out its processes, so studying the differences With the proposed methodology, it requires a different way of seeing the problem and the information obtained is the basis for the analysis of the complexity of the studied phenomena, with the obligation to see them in a non-reductionist way, to which we are used, for now. expectations exceeded our level of understanding

Conflict of Interests

The manuscript was prepared and reviewed with the participation of all the authors, who declare that there is no conflict of interest that could jeopardize the validity of the results presented

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