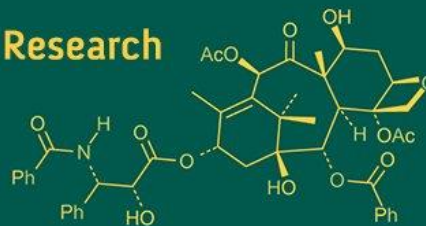


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Investigating the beneficial effects of co-culturing buffalo embryos with oviductal exosomes by evaluating the candidate transcripts of embryo conditioned medium

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Abstract

Achievement of successful pregnancy and high-quality offspring(s) with *in vitro* embryo production system always demands recreating a physiological environment. In the present study, the embryos were comparatively evaluated for their quality-determining transcriptomic signature by a relative analysis of the conditioned medium with embryo produced in the presence and absence of oviductal exosomes. Investigation on the relative abundance of mRNA transcripts showed a significant upregulation of the candidate genes: CX43 (related to compaction and cell-cell adhesion), SCD (related to lipid metabolism), GPX (protect against oxidative stress/ROS) and BCL2 (antiapoptotic transcripts) in the conditioned medium of exosome treated embryos. On comparative evaluation of the relative abundance of distinct miRNAs between the two study groups, upregulation of miR-184 (associated with viable embryos) and downregulation of miR-24-3p (associated with degenerate embryos) and miR-191 (associated with degenerate/aneuploid embryos) was noticed, which further substantiate the upswing in developmental competence and quality when embryos were cultured in the presence of oviductal exosomes. Bioinformatics analysis further supported the distinct role of the three miRNAs studied during embryo development, by regulating important biological pathways like Wnt/MAPK signaling. This study concluded that co-culture with oviductal exosomes significantly improves the beneficial gene expression pattern of bovine embryos, by providing the conducive maternal environment required for early embryonic development.

Keywords: Exosomes, co-culture of embryos, developmental competence, transcriptome

1. Introduction

In the *in vitro* embryo production system, embryos totally miss out on the early oviductal life as the oviduct nurtures the conditions required for sperm capacitation, transport, and fertilisation of the matured oocyte until early embryonic development. Additionally, the epigenetic modification that modulates the embryo development and further implantation also happens while the embryo resides in the oviduct. At the molecular level, embryonic genome activation (EGA) to overcome the developmental arrest at the 8-16 cell stage, also takes place while it is in the oviduct (Graf *et al.* 2014) [7]. Thus, the provision of an oviductal like environment is the missing link during *in vitro* embryo production making the outcome inferior to the *in vivo* physiological system.

Several *in vitro* models have been suggested to mimic the oviductal environment, which includes co-culture with oviductal epithelial cells (OECs) (Cordova *et al.* 2014) [4] and the use of oviductal fluid (OF) (Lopera-Vasquez *et al.* 2016) [15, 17]. The methodological complexity, associated bio-sanitary risks, and lack of reproducibility associated with using OECs and OF demand an alternative model. Beyond understanding traditional cell-to-cell communication, recent researches invited attention to the membrane-limited nanovesicles secreted by cells, called extracellular vesicles (EVs). By transferring the biomolecule(s) it carries (mRNA, miRNA, and proteins), EVs modulate the activities of recipient cells by participating in active intercellular communication, playing a key role in the regulation of important physiological and pathological processes (Ni *et al.* 2020) [24].

Oviductal exosomes can be a potential substitute for OECs and OF for *in vitro* embryo co-culture systems and can be derived from both sources. Studies by Lopera-Vasquez *et al.* (2016) ^[15, 17] evidenced that, exosomes derived from the OEC-conditioned medium improved the quality of bovine blastocysts produced *in vitro*, to the same extent as done with OEC monolayers.

The effect of exosome supplementation on the development and quality of *in vitro* produced embryos can be assessed in different ways. Invasive methods like differential staining of embryos can be attempted to evaluate embryo development based on the number of cells of the inner cell mass (ICM) and the trophectoderm (TE) and for the viability of embryos (Thouas *et al.* 2000) ^[27]. A widely used non-invasive method to assess embryo development and quality is by assessing the embryo secretome of the conditioned medium. It is advantageous over the morphological selection of embryos which is strongly influenced by inter-observer variations and is associated with lower accuracy. Advances in omics technologies have enabled the application of new molecular methods for investigation into their transcriptomic and proteomic content of the embryo conditioned medium. Earlier researches reported that oviductal EV supplementation to embryo culture medium has an impact on gene expression (Lonergan *et al.* 2006) ^[14], miRNA transcriptome (Kropp *et al.* 2014) ^[12] and proteome (Banliat *et al.* 2022) ^[3], consequently determining

embryo quality.

With the above background knowledge, the proposed study evaluated the differential expression of candidate RNAs (mRNAs and miRNAs) during *in vitro* culture of buffalo embryos when cultured alone and in the presence of oviductal exosomes.

2. Materials and Methods

To evaluate the potential benefits of oviductal exosomes, buffalo embryos were produced in the presence and absence of oviductal exosomes *in vitro*. The exosomes supplemented in the study were earlier isolated from OEC culture conditioned medium and was characterised following MISEV (minimum standard for extracellular vesicles) recommendations (Naheef *et al.* 2024b) ^[22, 23]. On Day 6 post-fertilization, the developmental status of the embryos was examined under a stereo zoom microscope, and the embryos were removed from the culture wells in a minimal amount of medium. The remaining embryo-conditioned medium (ECM) was harvested and utilized for molecular evaluation of candidate mRNAs and miRNAs. An equal number of exosomes that are supplemented in the exosome co-culture group was added to the ECM collected from the control group (embryo culture without oviductal supplementation) to nullify the effect of molecular cargo provided by oviductal exosomes.

Table 1: Primers used for detection of mRNA transcripts by qRT-PCR

Sl. No	Gene target	Primer sequence	Reference
1	bta-miR-24	TGGCTCAGTTCAGCAGGAACAG	Kropp and Khatib, 2015 ^[11]
2	bta-miR-191	CAACGGAATCCCAAAAGCAGCTG	Kropp and Khatib, 2015 ^[11]
3	bta-miR-184	TGGACGGAGAACTGATAAGGGT	Mellisho <i>et al.</i> 2019 ^[19]

2.1 Expression profiling of candidate mRNAs in the ECM

Total RNA was isolated from all the biological replicates of the ECM, following the Trizol (RNAiso Plus, Takara) based protocol. Immediately after extraction and quantification, the purified RNA was reverse transcribed to cDNA using PrimeScript RT reagent kit (Takara). Quantitative Real-time PCR (qRT-PCR) was performed to quantify the expressed mRNA levels for the selected genes (GPX1, SCD, CX43 and BCL2). All the qPCR reactions were performed using TB Green Premix ExTaq (Takara). The annealing temperatures used were: 60 °C for GPX1 and SCD; 56 °C for CX43 and 62 °C for BCL2. Primers with the published sequence as mentioned under Table 1 were used for amplification. Relative expression levels of each gene studied under exosome treatment and control group were quantified by the comparative cycle threshold method ($\Delta\Delta C_T$) method. Values were normalised using H2AFZ (Histone) as housekeeping gene. The normalised values

were subjected to statistical analysis and the means were compared by unpaired t-test using GraphPad Prism 9.5.1 at 1% level of significance.

2.2 Expression profiling of candidate miRNAs in the ECM

Component miRNA transcript was isolated from the conditioned medium of each biological replicate using NucleoSpin miRNA isolation kit (Macherrey Nagel) and reverse transcription was achieved using the miRNA first strand synthesis kit (Takara). The expression levels of candidate miRNAs were quantified using TB Green Advantage qPCR mix (Takara) in the CF-96 qPCR detection system (BioRad). As per the manufacturer guidelines, the expression level of each miRNA was determined relative to the level of U6snRNA. The qPCR protocol used designed 5' primers specific for different miRNAs of interest (Table 2) and a universal 3' primer provided by the manufacturer.

Table 2: Sequence of primer (forward) used for qPCR detection of candidate miRNA transcripts (Universal reverse primer (mRQ 3') provided in the kit was used in the first strand cDNA reaction protocol).

mRNA target	Primer sequence	Ampliqon size	References
SCD	TGGCTTTTCTCTCTCACG TACCTCCTCTGGAACATCAC	126 bp	Cordova <i>et al.</i> 2014 ^[4]
CX43	TGGAATGCAAGAGAGGTTGAGG AACACTCTCCAGAACACATGAG	293 bp	Lonergan <i>et al.</i> 2003 ^[13]
BCL2	GCCGAGATGTCCAGTCAGC GACGCTCTCCACACACATGAC	150 bp	Qu <i>et al.</i> 2019
GPX1	CCTCAAGTACGTCCGACCTG CAATGTCGTTGCGGCACACC	230 bp	Cordova <i>et al.</i> 2014 ^[4]
H2AFZ	AGGACGACTAGCCATGGACGTG CCACCACCAGCAATTGTAGCCG	208 bp	Lopera-Vasquez <i>et al.</i> 2017 ^[16]

A real time PCR reaction for U6snRNA was separately performed for each cDNA sample analysed. The qPCR was performed for each sample in duplicates, and the $2^{-\Delta\Delta Ct}$ method was used to analyse the data, as done for the relative mRNA expression described earlier.

2.3 Bioinformatics analysis of the differentially expressed miRNAs

For the identification of biological pathways, bioinformatics analysis of the identified miRNAs that were differentially expressed between the treatment and control groups was carried out. The targets of miRNAs were predicted using TargetScanHuman v 8.0 and miRWalk following default parameters. For downstream analysis, target genes predicted by TargetScanHuman v 8.0 were used. The predicted target genes were subjected to gene ontology and pathway mapping using DAVID. The GO terms and pathways were sorted on fold enrichment values. The analysis was conducted with a default EASE score of 0.1 to include most terms.

3. Results

To support the findings that supplementing oviductal exosomes during *in vitro* embryo culture improved the developmental rate and viability of buffalo embryos, molecular investigation into the differential expression of

candidate mRNAs and miRNA transcripts in the ECM was carried out.

3.1 Differential expression profiling of candidate mRNAs in the ECM

The relative expression levels of selected gene transcripts that were already been proven to have a crucial role in embryo development and quality were analysed by qRT-PCR. The genes selected in the study were: CX43 (related to compaction and cell-cell adhesion), SCD (involved in lipid metabolism), GPX1 (involved in protection against oxidative stress/ROS), and BCL2 (anti-apoptotic transcripts). Histone gene (H2AFZ) was taken as the housekeeping gene in the study.

Relative expression of the genes between the exosome treated and control group was represented as fold change, using $2^{-\Delta\Delta Ct}$ method. It was found that all the candidate genes studied were upregulated in the conditioned media transcriptome of embryos produced in the presence of oviductal exosomes. The relative abundance of each gene was statistically analysed by unpaired t-test and the results are represented in Figure 1. The fold change in expression (given in brackets for each candidate gene) was: CX43 (39.06), GPX1 (17.10), SCD (12.74), and BCL2 (8.70). All the results were statistically significant ($p \leq 0.01$).

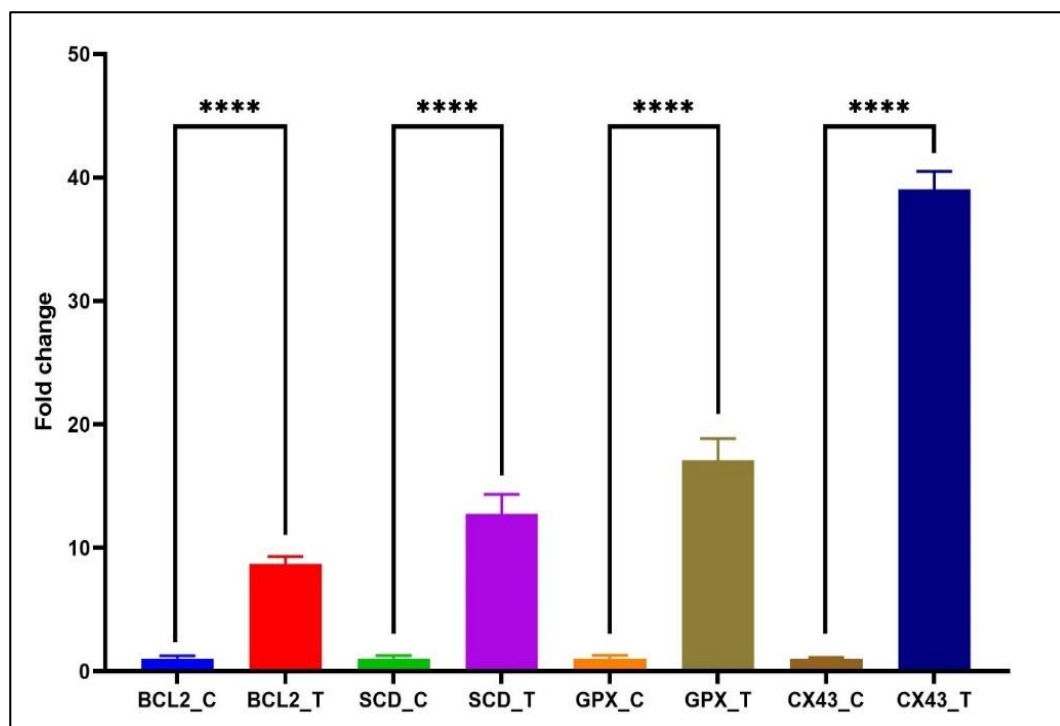


Fig 1: Relative expression levels of candidate mRNAs in the ECM ('T' stands for exosome treated group and 'C' for control group embryos cultured without exosomes)

3.2 Differential expression profiling of candidate miRNAs in the ECM

Among a large number of miRNAs cited as crucial for embryo development, three important miRNAs differentially expressed between exosome-treated and control group embryo-conditioned medium were statistically validated by real-time PCR. The miRNAs selected in the study were: bta-miR-24-3p, bta-miR-191, and bta-miR-184. Alongside, U6snRNA was chosen as the internal reference. The fold change in expression for each of these miRNA

transcripts studied under the exosome-treated and control group evaluated by $2^{-\Delta\Delta Ct}$ method is presented in Figure 2. Real-time PCR analysis revealed that both miR-24-3p (9.45-fold) and miR-191 (8.05-fold) were downregulated when exosomes were supplemented during *in vitro* embryo culture. The expression of miR-184 was found to be upregulated by 13.19-fold in the presence of oviductal exosomes in the culture. The fold change in the relative abundance of the transcripts studied was analysed by unpaired t-test and was statistically significant ($p \leq 0.01$).

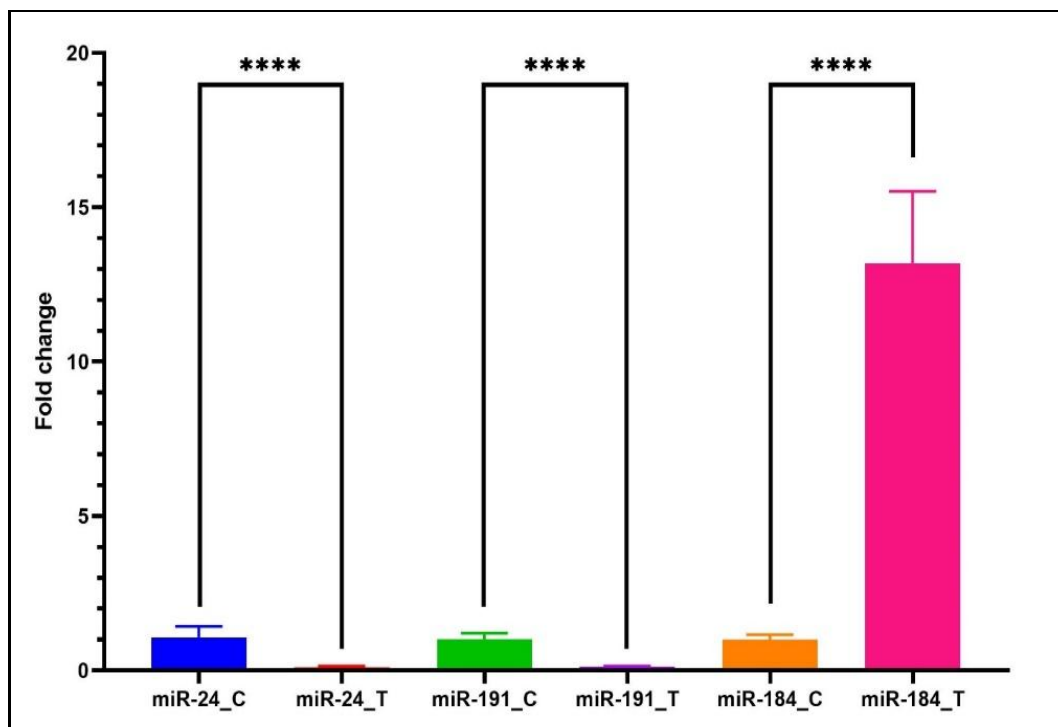


Fig 2: Relative expression levels of candidate miRNAs in the ECM ('T' stands for exosome treated group and 'C' for control group embryos cultured without exosomes)

3.2.1 Bioinformatics analysis of the candidate miRNAs

Functional analysis of the candidate miRNA target genes was carried out to identify the associated signalling/biological pathways. Among a large number of pathways identified for each miRNA, a few were selected based on their importance in the study and based on fold

enrichment of the genes.

It was observed that pathways predicted for miR-24-3p included cytosolic transport, DNA repair, and regulation of prime metabolic processes, among many illustrated in Figure 3.

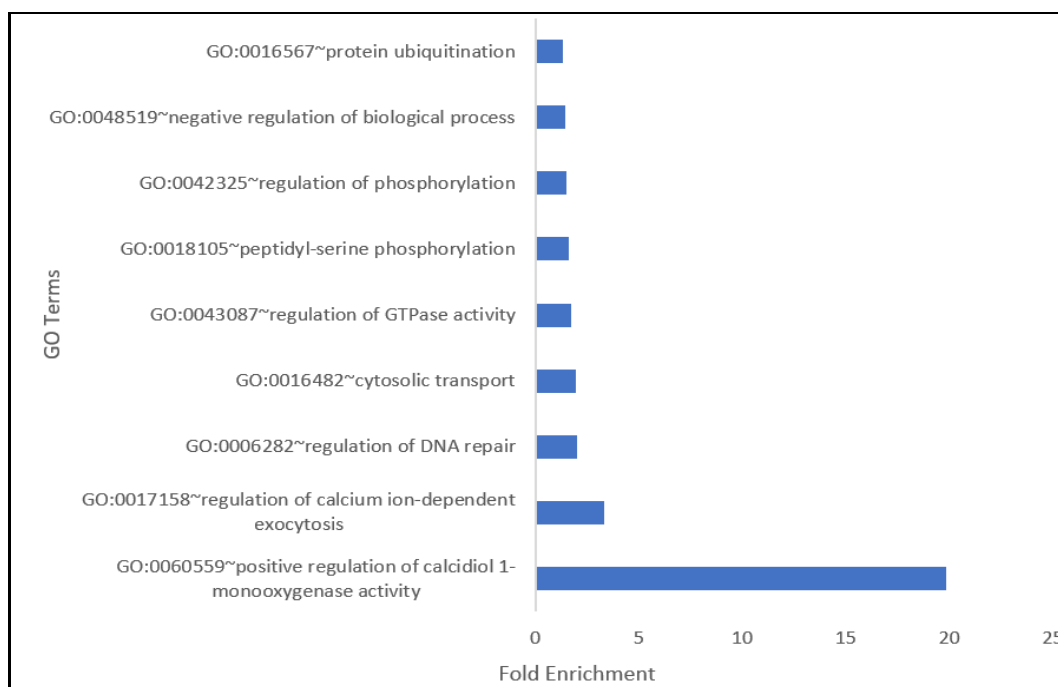


Fig 3: Important signalling pathways involving the target genes of miR24-3p with their respective fold enrichment values (Analysed by DAVID v2023q3 software)

The list of important pathways identified to be regulated by miR-191 included regulation of cellular process,

developmental process and cell morphogenesis as indicated in Figure 4.

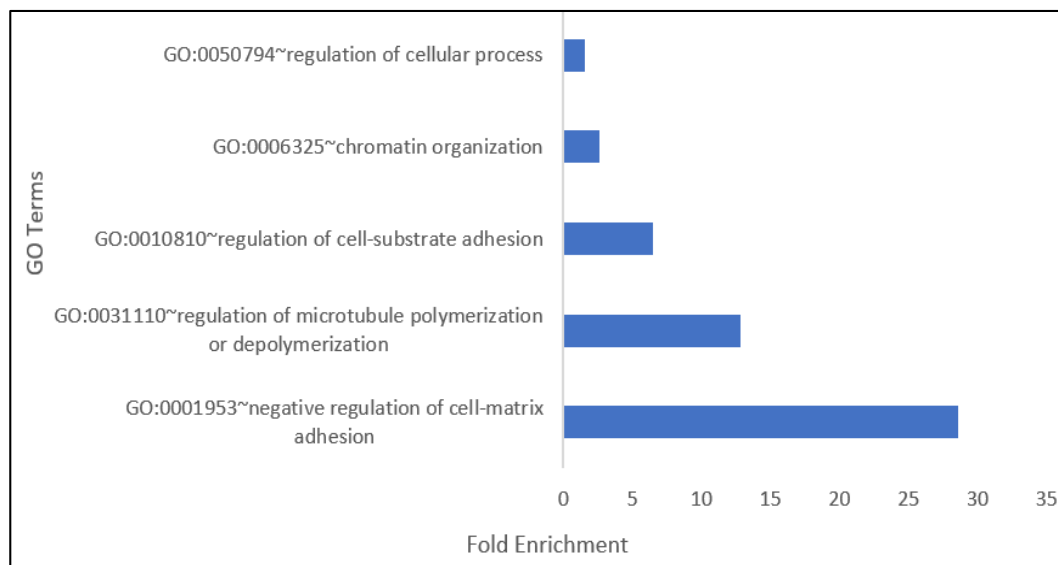


Fig 4: Important signalling pathways involving the target genes of miR-191 with their respective fold enrichment values (Analysed by DAVID v2023q3 software)

The important pathways identified revealed that miR-184 is involved in regulation of gene expression, cellular metabolic

and molecular processes, other biological events as represented in Figure 5.

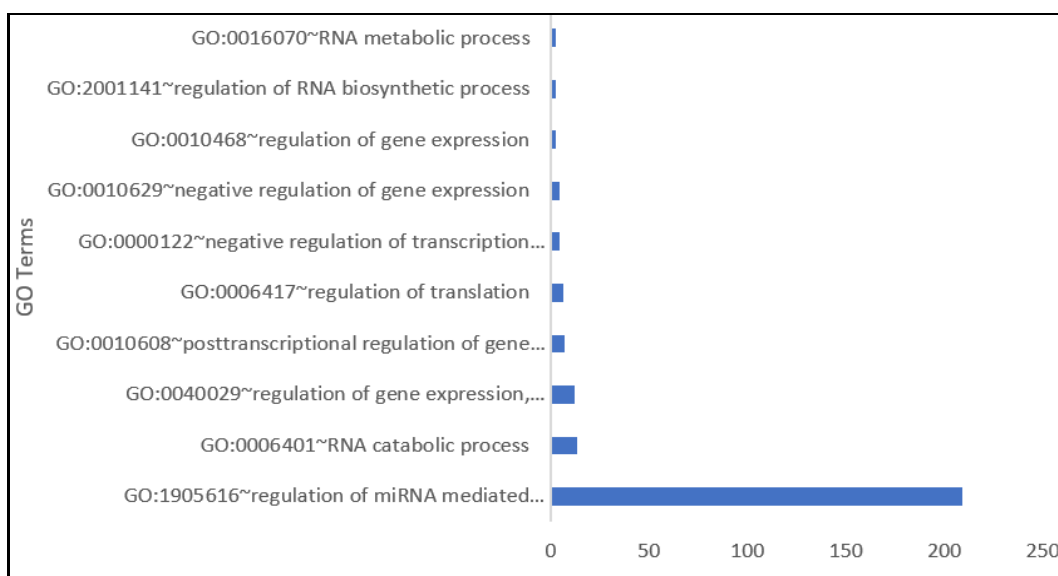


Fig 5: Important signalling pathways involving the target genes of miR-184 with their respective fold enrichment values (Analysed by DAVID v2023q3 software)

All the selected biological/signalling pathways and their functional clusters were found to be statistically significant ($p \leq 0.05$).

4. Discussion

With motives from previous reports in bovines, other domestic species, and humans, we have reported that embryo developmental rate and viability rate improved significantly when co-cultured with oviductal exosomes *in vitro* (Naheef *et al.* 2024a) [22, 23]. The present study attempted to correlate the data of microscopic evaluation with molecular evidence at transcriptomic (mRNA and miRNA) levels by investigating the conditioned media collected when embryos reached the morula/early blastocyst (EB) stage. To the best of our knowledge, this is the first study to validate the relative abundance of transcripts (mRNAs and miRNAs) upon supplementing oviductal exosomes during *in vitro* production of buffalo embryos.

4.1 Differential expression profiling of candidate mRNAs in the ECM

Even with an extensive investigation on the expression levels of several important genes, to date, consensus could not be attained in finalising the genes that would be potential markers for embryo development/quality. The genes transcripts selected in the study namely CX43, SCD, GPX1, and BCL2 are related directly to embryo development and quality.

Connexin 43 (CX43) plays a crucial role in embryo compaction and cell-to-cell adhesion. This study reported an upregulation of the CX43 transcripts up to 39.06-fold in the exosome-supplemented group as depicted in Figure 1. A similar finding was reported by Lopera Vasquez *et al.* (2016) [15, 17], who studied the expression of CX43 upon oEV (oviductal extracellular vesicle) supplementation during *in vitro* production of bovine embryos. Further support to the findings was provided by Cordova *et al.* (2014) [4], who

evidenced an upregulation of CX43 transcripts while bovine embryos were co-cultured with OEC monolayers *in vitro*. The upregulation of this particular gene is well documented in many *in vitro* studies, as it is expressed throughout the 8-16 cell stage of embryo development, both under *in vitro* and *in vivo* conditions (Lonergan *et al.* 2003)^[13] in bovines. Also, a higher expression of CX43 is generally associated with the presence of better-quality embryos having improved ability to tolerate cryopreservation and subsequent thawing as quoted by Gutierrez-Adan *et al.* (2004)^[8].

GPX1 is well understood for its role in fighting against oxidative stress and ROS-mediated developmental arrest of embryos. The present study revealed an increased expression level of GPX1 transcripts upon providing oviductal exosomes during *in vitro* embryo development. Similarly, Lopera Vasquez *et al.* (2016)^[15, 17] reported an upregulation of GPX1 gene upon utilizing the BOEC-derived exosomes for bovine embryo development. Functionally, GPX1 is related to free radical scavenging and contributes to mitigation of oxidative stress (Sidrat *et al.* 2020)^[26]. Thus, upregulation in GPX1 transcripts reflects the positive effect of exosome on embryo development by way of modifying the culture environment. Another possible explanation provided by Melka *et al.* (2010)^[18] is that OECs evade apoptosis of blastocysts through downregulation of TP53, a pro-apoptotic gene and that GPX1 gene is under the control of TP53.

SCD gene codes for primary enzymes involved in lipid metabolism. It can be inferred from Figure 1 that, SCD transcripts were upregulated in the conditioned medium of embryos produced by oviductal exosome-based co-culture system. This is in accordance with the reports of Cordova *et al.* (2014)^[4] who evaluated the merits on *in vitro* embryo development with the provision of BOEC monolayers during various stages of embryo development. Al Darwich *et al.* (2010)^[2] were able to correlate the increased expression of SCD gene in *in vivo* system with culture of embryos in sheep oviduct. He reported that GPX1 expression was higher in pure *in vivo* system, compared to embryos co-cultured with BOEC *in vitro*. This gene is also reported to improve survival rate of embryos upon cryopreservation and thawing, as the higher lipid content in trophoblast (TE) cells renders them more susceptible to damage during cryopreservation. As the oviductal epithelium is actively involved in lipid metabolism, these functions are thought to be mimicked by *in vitro* cultured BOECs (Cordova *et al.* 2014)^[4], and in turn its component exosomes. Although there are no earlier reports of using oEVs as such to validate its potential role during *in vitro* embryo development, all this scientific information positively correlates with the outcome of the current study. Being a proven antiapoptotic gene, the transcripts of BCL2 were selected in the study, to evaluate the beneficial effects of oviductal exosomes during *in vitro* embryo development. As shown in Figure 1, BCL2 transcripts were more abundant in the conditioned medium when embryos were co-cultured with oviductal exosomes. With OF based coculture system, Qu *et al.* (2019)^[25] have evaluated the functional importance of BCL2 (along with the proapoptotic gene Bax), in deciding the fate of *in vitro* produced embryos. They reported an upregulation of BCL2 and corresponding downregulation of Bax gene. Also, a ratio of the relative expression of BCL2 to Bax is supposed to give a better understanding of the survivability of embryos. Later, Fang

et al. (2022)^[6] in their study on porcine embryonic development with OEC-derived EVs provided supporting results with an upregulation of BCL2 during early embryonic development *in vitro*. Being a functional cargo, the functional effects brought about by the OEC/OF is supposed to be conserved with its constituent exosomes, producing the same effect.

4.2 Differential expression profiling of candidate miRNAs in the ECM

Micro RNAs (miRNAs) are the non-coding RNAs that modify biological processes either negatively by hindering translational repression or positively by targeting specific gene promoters. Aiming towards finding a miRNA-based non-invasive biomarker for embryo selection, the relative expression levels of three selected miRNAs (miR-24-3p, miR-191 and miR-184) were investigated in this study. U6snRNA was selected as the housekeeping gene in the study, as it was previously shown to be stably expressed in various developmental stages of bovine embryos (Abd El Naby *et al.* 2013)^[1].

In the present study, as represented in Figure 2, it could be seen that miR-24-3p transcripts were found to be downregulated when oviductal exosomes were supplemented during their *in vitro* development. Earlier studies of Kropp and Khatib (2015)^[11] evidenced a reduction in the expression of miR-24-3p transcripts in the conditioned medium of *in vitro* produced good quality bovine blastocysts, wherein an upregulation of miR-24-3p was observed up to morula stage and downregulation was noticed thereafter. Further, by supplementing miR-24 mimic in the embryo culture medium, they were successful in relating its increased expression with the presence of degenerated embryos. Thus, it is evident that downregulation of miR-24-3p is correlated to good quality, progressively developing embryos. The mechanism of action of this miRNA, is attributable to: (i) downregulation of dihydrofolate reductase, the S-phase enzyme involved in regulation of cell cycle (Mishra *et al.* 2009)^[20], and (ii) repressed expression of its target gene, CDKN1b, a cell cycle inhibitor (Kropp *et al.* 2014)^[12]. Further, Kasper *et al.* (2017)^[9] were able to correlate the down regulation of miR-24-3p with stress, experienced during *in vitro* culture of embryos.

In the present study, evaluation of miR-191 expression was also resulted to be downregulated when the embryos were co-cultured with oviductal exosomes, as represented in Figure 2. Kropp and Khatib (2015)^[11] positively related miR-191 expression with degenerated and aneuploid bovine embryos. Supporting the findings of the present study further, Weber *et al.* (2010)^[28] reported a higher expression of miR-191 under stressful conditions of human embryo development. Additionally, they found a relatively higher miR-191 expression in the medium of embryos fertilised by ICSI (intracytoplasmic sperm injection), possibly due to the physical damage produced to zona and oolemma during the procedure.

In this study, miR-184 was found upregulated when embryos were cultured with oviductal exosomes. This particular miRNA has not been much studied so far for their expression in bovine/human embryos. To date, there are no direct reports correlating the provision of oEVs (or its source of origin) and the resulting increment in the expression of miR-184. However, circumstantial evidence is

provided by Mellisho *et al.* (2019) ^[19] who reported an upregulation of miR-184 in viable bovine embryos, compared to non-viable ones. Similarly, the observations of low levels of miR-184 in maternal blood and associated early pregnancy loss in cattle embryos have been documented by De Bem *et al.* (2017) ^[5]. Reports of Zhang *et al.* (2019) ^[29] on the involvement of miR-184 in embryo resorption in mice and humans, also add on to our finding that miR-184 is upregulated in progressively developing embryos.

4.3 Bioinformatics analysis of the candidate miRNAs

Evaluation of the miRNAs using bioinformatics tools is valuable to identify their potential role in regulating biological processes or cell signalling. To gain insight into the regulatory role of the three miRNAs investigated in this study, relevant pathways and their target genes were predicted, as represented in Figures, 3-5. A detailed list was provided as Annexure. The Wnt and TGF β cascades were the most prominent among the signalling pathways predicted for these three miRNAs studied. These pathways were reported to have importance in the regulation of biological events essential for the establishment and maintenance of pregnancy. Particularly, Wnt signalling is conserved within the mammalian species, and it regulates various events in embryonic development including the determination of cell fate, motility, polarity, and organogenesis (Komiya and Habas, 2008) ^[10]. In mammals, TGF β ligands are also known to be important in the establishment and maintenance of early embryonic development and pregnancy (Monsivais *et al.* 2017) ^[21].

5. Conclusion

To evaluate the quality influence of oviductal exosomes during *in vitro* production of buffalo embryos and to go further towards choosing a non-invasive biomarker for embryo quality, analysis of candidate mRNA and miRNA transcripts of the embryo-conditioned medium (ECM) was performed. A higher expression was achieved for all the studied genes (CX43, SCD, GPX1, and BCL2) when exosomes were supplemented during embryo culture, indicative of the creation of a favourable new environment for *in vitro* development. Of the miRNAs studied, miR-24-3p and miR-191 were found to be downregulated at morula/EB stage when oviductal exosomes were supplemented during embryo culture. Another marker for embryo viability, miR-184, was found to be upregulated in the exosome co-cultured group. Thus, the results of mRNA and miRNA expression analysis supported the positive role played by oviductal exosomes during embryo development. Further bioinformatics analysis of the candidate miRNAs predicted the possible biological pathways, supporting the findings of this study.

6. Author Contributions

This work is a part of Ph.D thesis of Naheef, K. The work is carried out at Department of Animal Biotechnology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, India under the guidance of K. Brindhya, TMA Senthil Kumar, PSL Sesh, S Rangasamy and M Parthiban. VN Muhasin Asaf has contributed in the bioinformatics analysis performed in this work. All authors have read and agreed to publish the manuscript.

7. Conflicts of Interest

The authors declare no conflicts of interest.

8. Data availability statement

The data that supports the findings of this study are available from the first author upon reasonable request.

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