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Expression of HSF1 and GDF9 Genes in Bovine Oocytes Supplemented with Follicular Fluid Exosomes During *in vitro* Maturation

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ABSTRACT

The efficiency of embryo production using in vitro technologies in cattle remains suboptimal, with a substantial proportion of female gametes failing to develop after in vitro manipulation. As oocyte quality critically influences embryo development, identifying genetic markers to predict oocyte competence is vital. Follicular fluid-derived exosomes, which contain biomolecules that influence gene expression in oocytes, which enhances the oocyte developmental competence. This study, evaluated 318 culture-grade cumulus-oocyte complexes (COCs) collected from slaughterhouse ovaries and subjected them to in vitro maturation (IVM) under two groups. Group I (control) comprised 160 oocytes matured under standard physiological conditions (38.5°C) for 24 h, while Group II included 158 oocytes matured with follicular fluid exosome supplementation. Both groups were incubated in a controlled environment with 5 per cent CO_2 and 95 per cent relative humidity. The cumulus expansion rates were 92.73 ± 4.42% in Group I and 94.05 ± 2.62% in Group II, while polar body extrusion rates were 8.97 ± 2.72% and 10.98 ± 2.50%, respectively. Gene expression analysis revealed a significant down regulation of HSF1 (fold change: 0.84, $p \leq 0.05$) in Group II, whereas GDF9 showed an upregulation (fold change: 1.56), though not statistically significant. The findings suggest follicular fluid exosome supplementation may enhance oocyte maturation under invitro conditions.

Keywords: Bovine oocyte, Oocyte maturation, Exosome, HSF1 and GDF9.

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INTRODUCTION

Oocytes are highly specialised germ cells that develop within Graafian follicles and harbour half of the nuclear genetic material required for sexual reproduction. The follicular microenvironment comprises granulosa cells

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(GC) and follicular fluid (FF), which together create a dynamic milieu for oocyte growth and functional maturation. In mammals, oogenesis is a prolonged, multifaceted, and meticulously orchestrated process regulated by paracrine signalling pathways and intercellular junctional communications between the oocyte and its surrounding granulosa cells. This bidirectional communication is critical for modulating key cellular and molecular processes, including oocyte metabolic activity, cytoplasmic and nuclear maturation, as well as subsequent fertilization competence (Li and Albertini, 2013). Given the intricate nature of oogenesis, reproductive techniques have become critical in enhancing embryo production. Gamete competence is a primary determinant of success, influencing fertilization and subsequent embryo development. As a result, researchers are focused on identifying cellular and molecular markers to select competent oocytes and spermatozoa, thereby improving the efficiency and outcomes of reproductive technologies (Aguila *et al.,* 2020).

Supplementation of various chemicals to enhance the efficiency of embryo production has been extensively explored over the years. One of the most common components in maturation media is serum, which contains vitamins, amino acids, growth factors, cytokines, hormones, and other essential elements that support oocyte maturation and subsequent development. However, the use of bovine-derived sera or proteins is associated with a risk of contamination (Sagirkaya *et al.,* 2007). This highlights the need for alternative supplementation strategies and the optimization of culture conditions to improve the safety and efficiency of *in vitro* embryo production (IVEP).

The expression pattern of mRNA is valuable biomarkers for assessing the developmental potential of oocytes (Wrenzycki *et al.,* 1999). Extracellular vesicles (EVs), including exosomes, have attracted significant attention due to their role as nano-sized carriers of bioactive molecules. Present in various body fluids such as semen, epididymal fluid, endometrium, and follicular fluid, EVs influence key processes in both female and male reproductive systems (Kowalczyk *et al.,* 2022). A prominent component of follicular fluid, exosomes, regulate oocyte function by delivering cargo molecules through internalisation, interaction with cell membrane receptors, or endosomal pathways (Rodrigues *et al.,* 2019). Recently, mRNA expression patterns have emerged as valuable biomarkers for assessing the developmental potential of oocytes (Wrenzycki *et al.,* 1999). De Thonel *et al.* (2012) identified HSF1 as the most critical heat shock factor (HSF) in mammalian cells, serving as a key stress-responsive regulator. Under normal conditions, HSF1 exists in a latent state. The heat shock factor family, comprises four well-characterised transcription factors in mammals (HSF1, HSF2, HSF3, and HSF4) triggering the expression of genes encoding heat shock proteins. These proteins function as molecular chaperones, contributing to cytoprotection against various proteotoxic stresses and aiding in several pathological conditions. Growth Differentiation Factor 9 (GDF9), a critical member of the TGFβ superfamily, is an oocyte-derived paracrine factor essential for ovarian function. It regulates follicular development by promoting mitosis and differentiation of granulosa cells, thereby facilitating the formation and proliferation of the granulosa cell layers necessary for proper folliculogenesis (Paulini and Melo, 2011).

Exosomes present in semen, epididymal fluid, endometrium, and follicular fluid, EVs influence key processes in both female and male reproductive systems (Kowalczyk *et al.,* 2022). As a prominent component of follicular fluid, exosomes, regulate oocyte function by delivering cargo molecules through internalization, interaction with cell membrane receptors, or endosomal pathways. This study aimed to assess the effects of follicular fluid exosome supplementation on the IVM of bovine oocytes and to analyse the expression patterns of the HSF1 and GDF9 genes.

MATERIALS AND METHODS

Collection of ovaries and Isolation of exosomes

Bovine ovaries were obtained from local slaughterhouses and transported in thermos flasks filled with preheated normal saline supplemented with antibiotics to maintain a physiological temperature of 38.5°C. Within two hours of collection, upon arrival at the laboratory, extra ovarian tissues were trimmed, and the ovaries were thoroughly rinsed multiple times with 0.9 percent normal saline to minimise contamination. Exosomes were isolated from follicular fluid using differential ultracentrifugation. Initially, follicular fluid was collected and centrifuged at 500 × g for 1 min to remove debris. The supernatant was then stored at -80°C until a sufficient volume (15 mL) was available for further processing. The thawed follicular fluid was diluted with PBS and subjected to sequential centrifugations at 800 × g for 10 min and 2000 × g for 20 min to eliminate residual cells. The resulting supernatant was centrifuged at 12,000 × g for 45 mins to remove cellular debris and filtered through a 0.22 μm syringe filter.

To isolate extracellular vesicles, ultracentrifugation was performed at 110,000 × g for 70 min at 4°C (Sorvall WX Ultra Series Centrifuge (Thermo Scientific) equipped with a swinging-bucket Superspin Sorvall 30Ti rotor). The pellet containing exosomes was collected, and the superna

tant was discarded. The exosomal pellet was resuspended in 200 µL of filtered PBS, stored overnight, aliquoted, and preserved at -80°C for subsequent use.

Evaluation of In vitro maturation

Cumulus-oocyte complexes were retrieved by aspiration from 2–8 mm follicles and morphologically graded (Loos *et al.,* 1989). A total of 318 culture-grade COCs (Grades A and B, characterized by homogeneous ooplasm and at least three layers of cumulus cells) were randomly assigned to two groups for IVM. Group I (control) included 160 COCs cultured under standard IVM conditions at 38.5°C for 24 h, while Group II (experimental) included 158 COCs cultured under the same conditions with the addition of exosomes at a concentration of 1 µL per 100 µL of maturation medium. Both groups were cultured in modified Tissue Culture Medium-199 (mTCM 199) for maturation.

Oocyte maturation was assessed by the degree of cumulus cell expansion, as described by Kobayashi *et al.* (1992). The classification included: Degree 0 (slight or no expansion, cells tightly attached to the zona pellucida), Degree 1 (non-homogeneous expansion with clustered cells), and Degree 2 (homogeneous expansion with no clustering). The mean IVM rate (%) was determined by counting the number of oocytes exhibiting degree I and II cumulus expansion relative to the total number of oocytes cultured for IVM.0

Relative quantification

Following maturation, cumulus cells were separated from the oocytes by vortexing for 4 min and stored at -80°C in RNAlater (Sigma Lifesciences) until RNA extraction. Total RNA was isolated using the RNeasy Mini Plus kit (QIAGEN), according to the manufacturer's protocol. RNA samples from 40 A and B grade COCs were pooled, and RNA was eluted in 30 µL of RNase-free water. The concentration of eluted RNA was measured using a Nanodrop spectrophotometer, with most samples yielding approximately 10 ng/µL. The RNA concentration was normalized to 100 ng for cDNA synthesis by using 10 µL of the eluted RNA. cDNA synthesis was performed using a cDNA synthesis kit with RNase inhibitor (Origin Diagnostics, Kerala) and had an average concentration of 1000 ng/µL (Nano drop).

Primers for the target genes HSF1 and GDF9 were designed, with GAPDH as the reference gene. Gene expression levels were quantified using real-time PCR (qPCR) with the comparative C_T method. The qPCR conditions were optimised via gradient PCR, and the annealing temperatures for each gene were standardised. For qPCR, 1 µL of synthesized cDNA (1000 ng) was used per reaction, and reactions were performed in triplicate across three independent biological replicates.

Statistical analysis

Statistical analysis was conducted employing one-way ANOVA, with the threshold for significance established at $p < 0.05$ Kona *et al.* (2015). The analysis was performed using version 24.0 of the Statistical Product and Service Solutions (SPSS) software (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

Significance of follicular fluid exosomes

Follicular fluid (FF)-derived exosomes play a crucial role in oocyte maturation by serving as carriers of regulatory molecules, including proteins, microRNAs (miRNAs), and lipids. These small extracellular vesicles mediate intercellular communication between GCs and the oocyte, facilitating key reproductive processes. Exosomal miRNAs regulate signalling pathways critical for follicular growth and oocyte development, such as WNT, TGFβ, MAPK, and ErbB. Additionally, FF exosomes enhance cumulus-oocyte complex (COC) expansion by modulating the expression of genes such as Ptx3, Ptgs2, and Tnfaip6, while also protecting against GC apoptosis. Alterations in exosomal miRNA profiles due to aging and metabolic factors can compromise oocyte quality and fertilization potential. Emerging insights further highlight the connection between mitochondrial function and gonadotropin regulation, reinforcing the significance of FF exosomes in optimizing oocyte developmental competence (Pan *et al.,* 2024).

Evaluation of Cumulus cell expansion

In the present study, the mean ± SE of cumulus cell expansion was 92.73 ± 4.42 per cent in the control group and 94.05 ± 2.62 per cent in the exosome-supplemented group (Fig.1). Although there was a marginal increase in the cumulus expansion rates between the control and exosome-supplemented groups, the difference was not statistically significant. The high rate of cumulus expansion observed can be attributed to the careful selection of culture-quality oocytes (Grade A and B) for IVM. These oocytes were surrounded by at least three layers of cumulus cells, a feature strongly associated with greater develop-

mental competence. This observation aligns with findings by Nandi *et al.* (2002), who reported a higher degree of cumulus expansion in oocytes with more than three layers of cumulus cells, underscoring the importance of selecting high-quality oocytes for IVM.

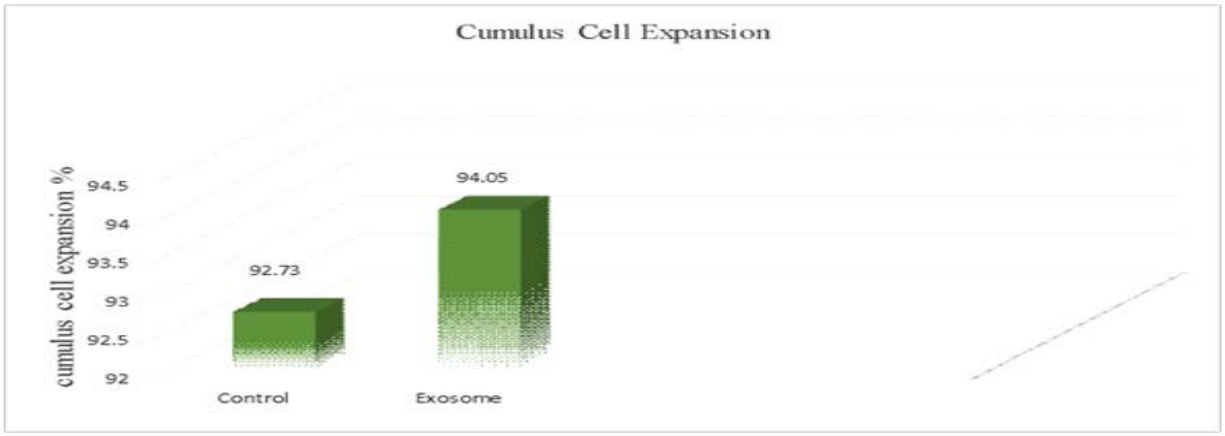

Fig. 1. Cumulus cell expansion in oocytes matured under normal physiological condition with and without exosome supplementation

Furthermore, exosomes derived from follicular fluid are known to carry molecular signals that enhance cumulus cell function and promote expansion. Hung *et al.* (2015) demonstrated that exosome supplementation increases the resilience of cumulus cells to environmental stressors, such as heat, by improving cell communication and restoring the expression of key genes involved in expansion. These findings signify the potential of exosome supplementation as a valuable tool for enhancing cumulus cell function and resilience, aligning with the observed marginal increase in cumulus cell expansion rates in this study and its implica tions for improving reproductive outcomes in assisted reproductive technologies.

First polar body extrusion

The mean percentage of first polar body extrusion in control and exosome supplemented groups were 8.97 ± 2.72 and 10.98 ± 2.50, respectively (Fig.2). However, the difference was not statistically significant. Studies conducted by Revathy *et al.* (2023) reported that exosome supplementation significantly influenced these processes, regardless of follicle size.

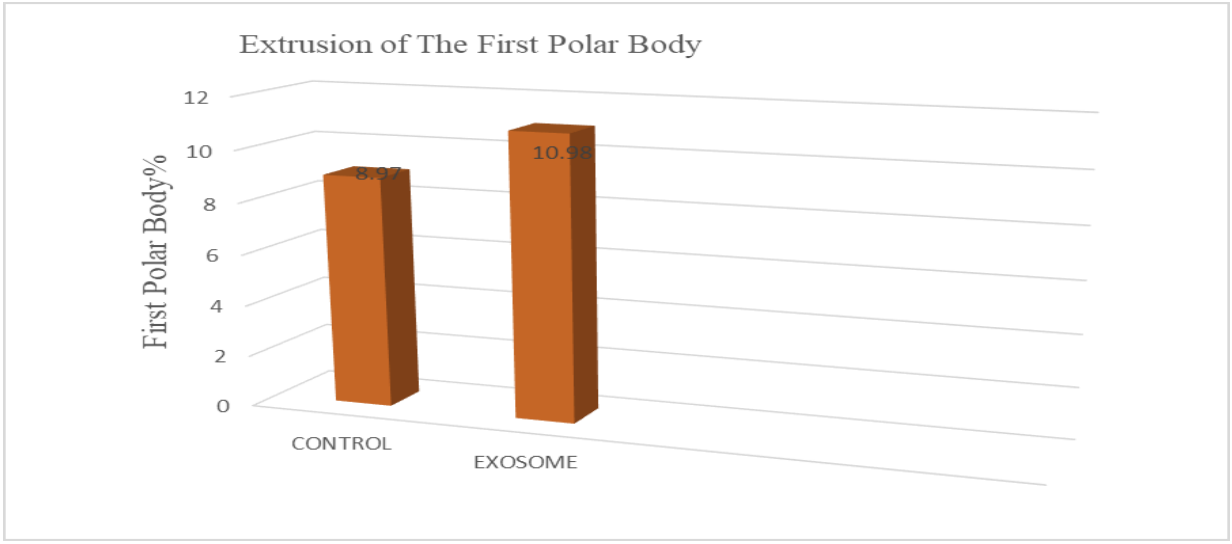

Fig. 2. First polar body extrusion in oocytes matured under normal physiological conditions with and without exosome supplementation

Relative Quantification of HSF1 gene expression in bovine oocyte

Heat shock factor 1 (HSF1) is a key regulator of the transcriptional program that governs cellular responses during stress. It plays a pivotal role in maintaining the dynamic expression of core proteostasis genes involved in the heat shock response (HSR). In the present study, HSF1 gene expression in the exosome-supplemented group (Group II) showed significant downregulation by 1.19-fold (fold change: 0.84) compared (Fig. 3) to the control group (Group I). Head *et al.* (1996) observed that HSF1 responds to heat shock as well as hypertonic stress caused by elevated potassium or sodium levels. Stress-inducible expression of heat shock proteins is predominantly regulated by HSF1. Under mechanical stress, HSF1 activation is mediated by the Ca^{2+}-Akt signalling axis. Intracellular Ca^{2+}enhances Akt activity, which phosphorylates and activates HSF1, linking mechanical and biochemical signals in the cellular stress response Du *et al.* (2023). Dalanezi *et al.* (2019) reported that HSF1 expression is reduced under thermoneutral conditions, consistent with our observations. The results were summarised in Table 1.

Table 1. Relative gene expression of HSF1 in bovine oocytes subjected to exosome supplementation

GROUP	Mean C_T ± SE		ΔC_T	$\Delta\Delta C_T$	Fold change($2^{-\Delta\Delta C_T}$)
	HSF1	GAPDH			
Group I Control	37.70 ± 0.45	38.30 ± 0.31	-0.61 ± 0.58	0	1^a
Group II Experimental	37.26 ± 0.80	37.61 ± 0.20	-0.35±0.645	0.26 ± 0.65	0.84^b ± 0.04 (1.19)

Means having different superscripts within a column differ significantly at p<0.01

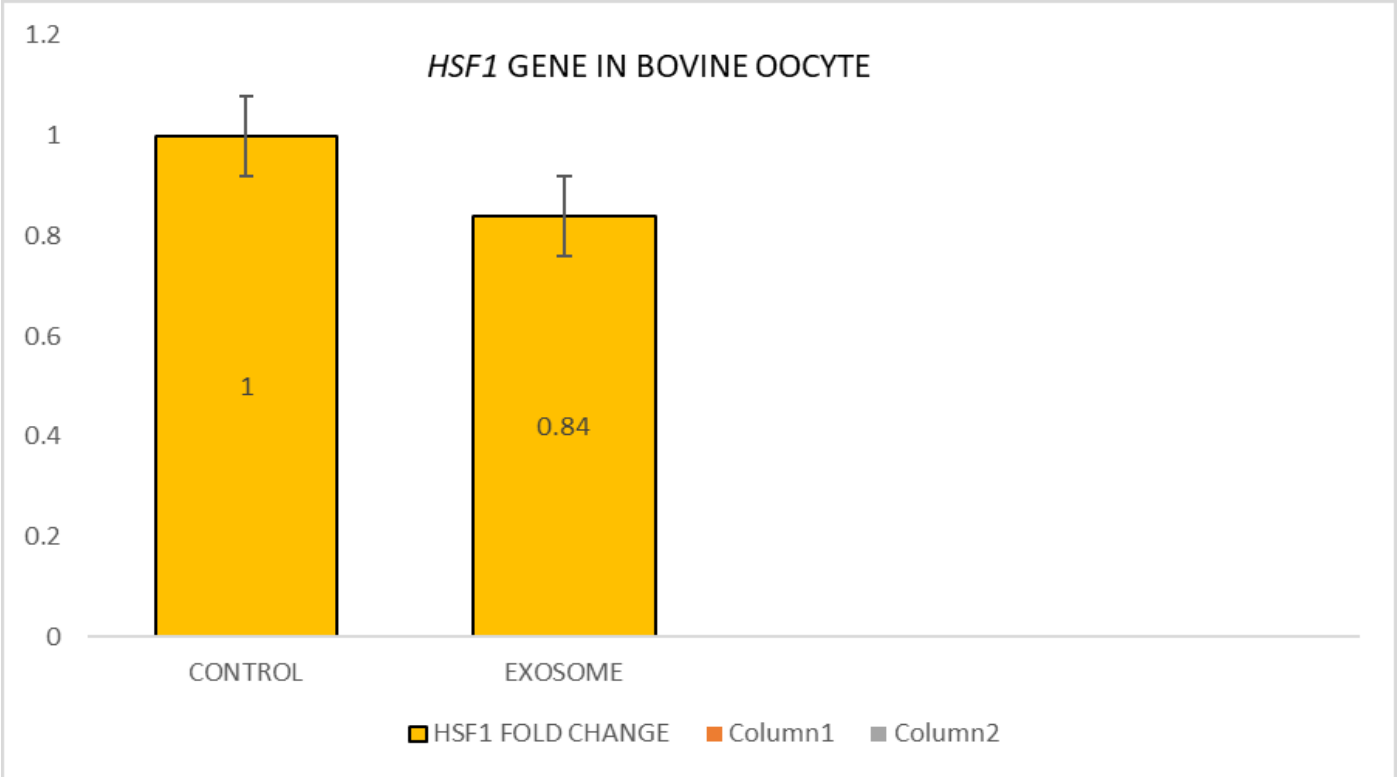

Fig. 3. Relative expression of HSF1 gene in bovine oocytes matured under normal physiological conditions

Relative Quantification of GDF9 gene expression in bovine oocyte

Growth differentiation factor 9 (GDF9*)* is an essential growth factor within the transforming growth factor-beta (TGF-β) superfamily, playing pivotal role in follicular development. Growth Differentiation Factor 9 and Follicle-Stimulating Hormone Receptor (FSHr) are pivotal molecular markers of cumulus cell functionality. These biomarkers play critical roles in directly facilitating oocyte developmental processes, inhibiting apoptotic pathways in cumulus cells, and creating an optimal microenvironment conducive to oocyte developmental competence and follicular growth (Azari *et al.,* 2017). In the current study, GDF9 gene abundance in Group II (exosome-supplemented group) showed upregulation by a fold change-1.56 compared (Fig. 4) to the control group. Wei *et al.* (2022) reported that treating oocytes with 3 per cent exosomes significantly upregulated genes related to oocyte maturation, including GDF9 and CPEB1, thereby enhancing the overall maturation process. Han *et al.* (2024) observed that exosome supplementation improved oocyte maturation, accompanied by increased endoplasmic reticulum activity and lipid droplet formation. Exosome supplementation influences GDF9 expression by delivering enriched microRNAs, such as miR-1246, miR-2904, and miR-374a, which regulate cellular stress pathways like heat stress, redox balance, and hypoxia, ultimately supporting enhanced oocyte development and function Gebremedhn *et al.* (2020). The results were summarised in Table 2.

Table 2. Relative gene expression of GDF9 in bovine oocytes subjected to exosome supplementation

GROUP	Mean C_T ± SE		Δ C_T	ΔΔ C_T	Fold change($2^{-\Delta\Delta C_T}$)
	GDF9	GAPDH			
Group I **Control**	36.56 ± 0.46	38.30 ± 0.31	-1.75 ± 0.60	0	1[a]
Group II Experimental	35.22 ± 0.24	37.61 ± 0.20	-2.31 ± 0.28	-0.65[b] ± 0.28	1.56[a] ± 0.60

Means having different superscripts within a row differ significantly at p<0.01

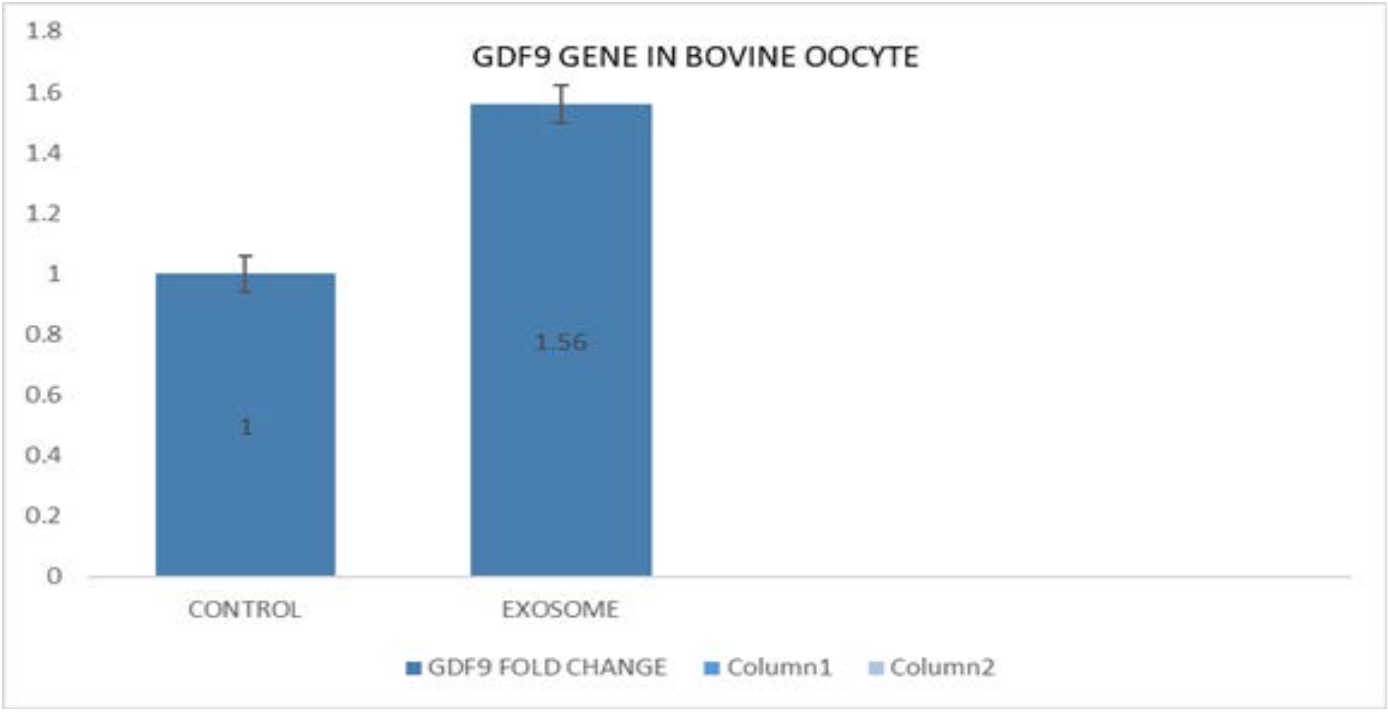

Fig. 4. Relative expression of GDF9 gene in bovine oocytes matured under normal physiological conditions

CONCLUSION

This study demonstrated that follicular fluid-derived exosome supplementation enhances the IVM of bovine COCs by improving cumulus expansion and polar body extrusion, essential for oocyte developmental competence. The downregulation of HSF1 and upregulation of GDF9 suggest exosomes mitigate cellular stress while enhancing oocyte-cumulus communication. As natural bioactive carriers, exosomes provide a physiologically relevant alternative to sera-based media, reducing contamination risks while supporting cytoplasmic and nuclear maturation, mitochondrial function, and steroidogenesis. By modulating key pathways in follicular growth and oocyte maturation, exosome supplementation offers significant potential to enhance in vitro embryo production (IVP) and improve assisted reproductive technologies.

CONFLICT OF INTEREST

Authors have no conflict of interest to declare

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