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Evaluation of the development and expression of BMP 15 and GDF 9 genes in sheep oocytes resulting from in vitro maturation and post-vitrification

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Abstract

The livestock sector plays a crucial role in food security; nevertheless, illnesses affecting cattle can result in genetic depletion and diminished output. This study sought to assess the expression of BMP-15 and GDF-9 genes in sheep oocytes undergoing in vitro maturation (IVM) and vitrification. Oocytes were extracted from ovine ovaries, developed in vitro, and subsequently vitrified. DNA was collected and measured, and subsequently subjected to qPCR analysis for BMP-15 and GDF-9 gene expression. The results demonstrated that DNA content in vitrified oocytes was considerably lower than that in non-vitrified controls, with higher Ct values reflecting decreased gene expression. The vitrification procedure probably induced oxidative stress and mitochondrial dysfunction, compromising DNA integrity and disrupting essential signaling pathways, hence diminishing BMP-15 and GDF-9 expression levels. The findings indicate that vitrification reduces gene expression associated with oocyte maturation, underscoring the necessity for enhanced vitrification techniques to maintain genetic potential in cattle reproduction. This study corresponds with the Sustainable Development Goals (SDGs), namely SDG 2 (Zero Hunger) and SDG 15 (Life on Land), by focusing on the necessity for novel reproductive methods to conserve cattle genetic resources. Enhancing vitrification techniques enables the preservation of genetic material essential for future breeding and resilience, thus promoting sustainable agricultural practices and contributing to long-term food security and biodiversity conservation.

Keywords: BMP 15, Food security, GDF 9, In Vitro Maturation, Vitrification.

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1. Introduction

The worldwide growth of the livestock sector is crucial for satisfying human demand for animal-derived goods, including meat, milk, and eggs. Nonetheless, advancements in the industry encounter considerable challenges, including illnesses affecting cattle health, productivity, and reproductive capabilities. Infected animals suffer reductions in output and, if not treated, may perish. Diseases such as avian influenza, SARS, foot-and-mouth disease (FMD), lumpy skin disease (LSD), peste des petits ruminants (PPR), and malignant catarrhal fever (MCF) have caused significant economic losses and adversely affected animal welfare in the livestock industry. Particularly in lethal instances, these illnesses lead to immediate investment losses and further destabilize supply networks and market equilibrium. Livestock fatalities result in less productive populations and a potential loss of significant genetic traits, especially devastating when the deceased animals exhibit superior genetics.

In Indonesia, foot-and-mouth disease, which was earlier proclaimed eliminated in 1986, re-emerged as an outbreak in 2022. Foot-and-mouth disease (FMD), induced by the RNA virus from the Picornaviridae family, affects susceptible animals predominantly via the respiratory system in cattle and the gastrointestinal tract in pigs [1]. As of March 2024, there were 620,534 documented cases of sick animals, resulting in 11,837 fatalities, 14,659 mandated culls, and 28,504,780 vaccinations administered. The most instances were seen in beef cattle (82.30%), followed by dairy cattle (11.80%) and buffalo (4.5%). The execution of the stamping-out or conditional culling strategy prompts apprehensions over population reduction and the possible depletion of vital genetic resources [2]. Lumpy skin disease (LSD), caused by a DNA virus from the Capripoxvirus genus within the Poxviridae family, is a non-zoonotic illness that affects cattle and buffalo [3]. LSD infection undermines production and reproductive efficacy, leading to decreased milk output, weight loss, abortion, infertility, and, in extreme instances, mortality.

Peste des petits ruminants (PPR) is a highly infectious transboundary animal disease (TAD) that primarily affects sheep and goats [4]. This illness, caused by a Morbillivirus from the Paramyxoviridae family, has elevated morbidity rates (90%) and fatality rates ranging from 30% to 70% [5], presenting significant challenges to cattle productivity in endemic regions. Malignant catarrhal fever (MCF) is an immunolymphoproliferative illness that impacts large ruminants and can infect a variety of animals, including cattle, buffalo, bison, pigs, deer, and horses. Malignant catarrhal fever (MCF), caused by the malignant catarrhal fever virus (MCFV) from the Rhadinovirus group and Herpesviridae family, is endemic in certain Indonesian areas, particularly in Bali cattle kept in proximity to sheep, which serve as carriers of the virus [6].

The elevated death rates linked to these illnesses necessitate the conservation of essential genetic resources, particularly in instances of cattle with exceptional genetics. Gamete preservation, encompassing spermatozoa and oocytes, constitutes an effective approach for retaining genetic material. The methods for preserving male gametes are straightforward and enable the effective storage of substantial amounts. Nonetheless, female gametes, particularly oocytes in the ovary, have restricted viability, impeding prolonged storage at ambient temperatures [7, 8].

Oocyte preservation is less prevalent than sperm or embryo preservation owing to its technological intricacies [9]. The restricted surface area and susceptibility of oocytes to chilling procedures pose problems [10, 11]. Recent developments in oocyte preservation have shown inconsistent results, with vitrification cryopreservation preferred for its cost-effectiveness and efficiency [12-14]. One method to extend the viability of oocytes entails the application of preservation strategies, followed by the modulation of in vitro developing settings. The cryopreservation of oocytes is intended to retain and sustain cellular viability, thereby protecting the integrity of the oocytes for future use in in vitro embryo development. The resulting embryos can then be transplanted to recipient animals or cryopreserved for future use [15].

In connection with the Sustainable Development Goals (SDGs), specifically SDG 2 (Zero Hunger) and SDG 15 (Life on Land), Indonesia confronts the dual challenge of enhancing its livestock sector while alleviating the impact of animal diseases on food security, economic development, and environmental sustainability. Improving animal health management, fortifying disease surveillance, and investing in genetic conservation are essential ways to decrease livestock mortality and maintain biodiversity, thereby supporting resilient agricultural systems. The conservation of genetic resources, including gametes, enables herd rebuilding during crises and maintains production, directly supporting SDG aims [16]. Sustainable practices in the livestock sector are essential for guaranteeing long-term food security, alleviating poverty, and maintaining ecological balance in both rural and urban areas in Indonesia [17].

The aim of oocyte cryopreservation is to preserve cellular viability for further application in in vitro embryo development. Mature embryos may be delivered to recipient animals or preserved for future utilization. In vitro oocyte maturation (IVM) is a vital phase in embryo creation, during which the secretion of growth hormones into the medium facilitates development [18]. The BMP-15 gene is crucial for proper follicular development and inhibits cumulus cell death by regulating local apoptotic gradients within cumulus-oocyte complexes (COCs).

The GDF-9 gene, a ligand of TGF-beta, is crucial in in vitro maturation (IVM). Vitrification techniques, meanwhile, diminish the expression of GDF-9 and BMP-15 genes [19]. The function of intra- and extracellular cryoprotectants is essential during vitrification to safeguard oocytes from oxidative stress, which escalates with significant temperature fluctuations, potentially resulting in mitochondrial dysfunction and impaired RNA and DNA synthesis, thereby diminishing the expression of genes such as GDF-9 and BMP-15 [20]. This study underscores the need for genetic preservation in female animals afflicted by illness, especially those necessitating culling or death. Preserved genetic material can facilitate future breeding efforts, which are essential for post-outbreak repopulation and for sustaining animal population stability and productivity.

2. Methodology

The research sample consists of oocytes obtained from the ovaries of sheep sourced from the abattoir. The stages of this research include oocyte collection, oocyte maturation, oocyte vitrification, RNA isolation, and PCR. This research has received ethical clearance with ethics number 1.KEH.147.10.2024.

2.1. Collection of Ovaries and Oocytes

Sheep ovaries were collected from a local slaughterhouse and transported to the laboratory in physiological saline (0.9%, w/v, NaCl) supplemented with 50 µg/ml gentamicin sulfate (Gibco, USA) at a temperature of 35°C within 4 hours.

Oocytes in the form of Cumulus Oocyte Complex (COC) were subsequently collected from the follicles in each ovarian group using the aspiration method in Dulbecco's Phosphate Buffered Saline (DPBS; Gibco, USA) media supplemented with 5% Fetal Bovine Serum (FBS; Gibco, USA) and 50 µg/ml gentamicin sulfate (Gibco, USA). COC is classified based on the number of cumulus cells surrounding it and the homogeneity of the cytoplasm. COCs with more than 4 layers of cumulus cells are rated as class 1, 3-4 layers are class 2, and 0-2 layers are class 3. Oocyte maturation is then carried out in maturation media supplemented with FSH and LH hormones. The oocytes are matured in an incubator at 37°C, 5% CO2, for 24-26 hours. The matured oocytes are then evaluated and counted as a percentage of the total number of matured oocytes.

2.2. Oocyte Vitrification

After maturation, the mature oocyte is marked by the appearance of polar body II. The identified mature oocyte is then preserved through vitrification. Oocytes are exposed to an equilibration medium containing 10% ethylene glycol and 0.5 M sucrose for 5-7 minutes. After the equilibration process is achieved, the oocytes are exposed to a cryoprotectant solution containing 15% DMSO, followed by 15% ethylene glycol and 0.5 M sucrose for 30 seconds. Finally, the oocyte is placed into a straw and frozen in liquid nitrogen (-196°C). The warming stages are carried out by exposing the oocytes to a graded sucrose solution, namely 0.5 M, 0.25 M, and 0.1 M.

2.3. Oocyte RNA Isolation and qRT-PCR

To perform genomic analysis, RNA is isolated using a commercial kit. Spermatozoa and oocyte samples were lysed using a lysis buffer according to the instructions provided by the kit manufacturer. Subsequently, RNA was stored at -20°C until it was used for genomic analysis in the qRT-PCR reaction. Oocyte RNA extraction was performed following the RNeasy mini kit instructions (Qiagen). Next, cDNA synthesis was performed using the RevertAid qPCR RT master mix with gDNA remover. The concentration of the obtained cDNA was measured using a Nanodrop and then stored until the quantification process was carried out.

2.4. Primer Design

Primers for gene expression detection were designed with the help of the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/), where gene information refers to the NCBI database (www.ncbi.nlm.nih.gov). The primary gene design used in this study can be seen in.

Table 1.
List of genetic marker primers.

Gene name	Accession number (NCBI)	Primer Sequence 5'-3'		Annealing Temperature (°C)	Product Size (bp)	Reference
GDF9	NM_001142888.2	F	GAAGACTGGTATGGG GAAATG	58	462	Kasiriyan, et al. [21]
		R	CCAATCTGCTCCTAC ACACCT			
BMP15	NM_001114767.2	F	CACTGTCTTCTTGTT ACTGTATTTCAAAC	62	141	Kasiriyan, et al. [21]
		R	GATGCAATACTGCCT GCTTG			
GAPDH	NM_001190390.1	F	GGCGTGAACCACGAG AAGTATAA	57	119	Lucas Nascimento Souza, et al. [22]
		R	CCCTCCACGATGCCA AAGT			
SDHA	XM_027980212.2	F	ACCTGATGCTTTGTG CTCTGC	57	126	Lucas Nascimento Souza, et al. [22]
		R	CCTGGATGGGCTTGG AGTAA			
RPL-19	XM_027974613.2	F	GAAATCGCCAATGCC AAC	51	361	Lucas Nascimento Souza, et al. [22]
		R	GAGCCTTGTCTGCCTTCA			

The qRT-PCR analysis on the specific gene was conducted with a program of 95°C for 1 minute, 95°C for 15 seconds, and 58°C for 1 minute, according to the manufacturer's instructions for the RT-PCR kit. In this study, the housekeeping genes used are GAPDH, SDHA, and RPL-19. The method for calculating gene expression uses the 2 Delta Ct (ΔCt) method, which involves calculating the difference between the target gene and the housekeeping gene: (2-ΔΔCt= [(Ct target gene) – (ΔCt housekeeping gene) sample] - [(Ct target gene) – (ΔCt housekeeping gene) standard] [23]. The results of gene expression

were calculated in terms of relative mRNA quantity and subsequently tabulated for statistical comparison between treatments [24].

3. Research Results

3.1. DNA Detection with Conventional PCR

The sample optimization was performed using traditional PCR, resulting in no visible bands in the ELP samples, whereas bands were seen in the control (M = marker; K+ = sheep meat DNA). The lack of bands in the ELP samples is likely due to inadequate DNA concentration (below 50 ng/µL), which impedes the amplification of the target DNA. Thus, the decision was made to utilize real-time PCR (qPCR) for the identification of BMP-15 and GDF-9 genes, ensuring a more sensitive and precise evaluation.

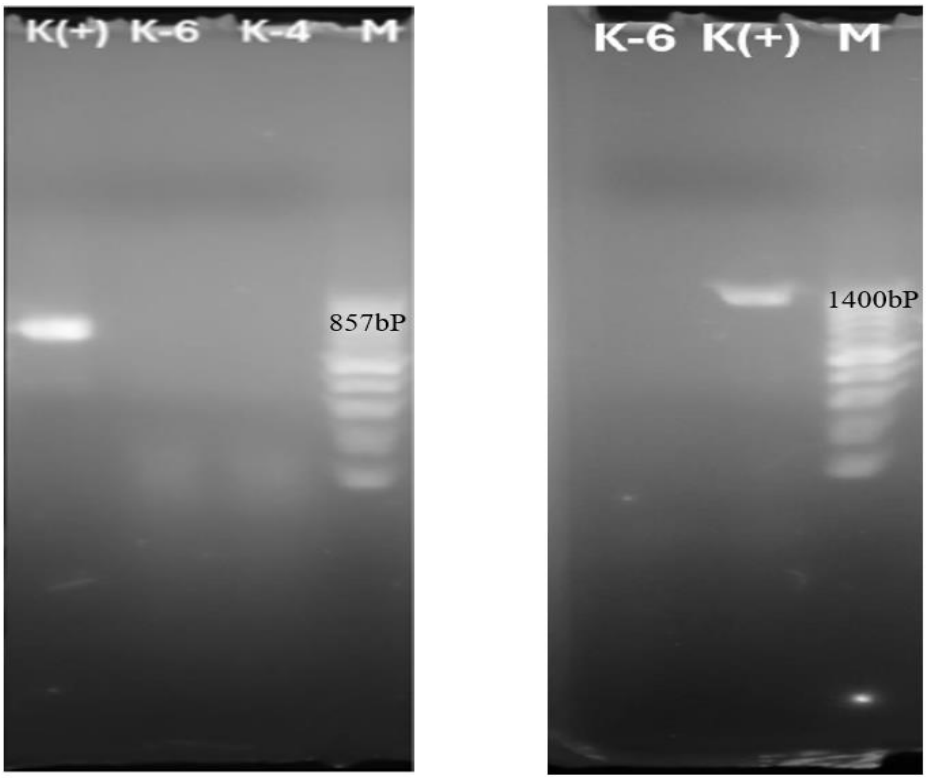

Figure 1.
Conventional PCR band of BMP-15 gene (left) and GDF-9 (right).

3.2. DNA Measurement

Table 2 displays the DNA measurement data for each analyzed gene, comprising two sample groups: the control group (C-negative) and the treatment group (T). The C-negative sample group experienced in vitro maturation (IVM) without vitrification, while the T group of oocytes received both IVM and vitrification. The initial DNA concentration of each sample group was assessed in total, with amounts varying from 43.5 to 81.3 ng/µL across individual samples.

Table 2.
Results of the overall DNA concentration measurement in goat oocytes after maturation and post-vitrification.

Sample code	DNA concentration of BMP-15 sample group (ng/µl)	DNA concentration of GDF-9 sample group (ng/µl)	Average DNA concentration (ng/µl)
C-1	59.3	103.3	81.3
C -2	66.6	69.0	67.8
C -3	79.4	71.6	75.5
C -4	56.6	67.1	61.9
C -5	63.1	67.1	65.1
C -6	71.6	56.6	64.1
C -7	71.6	78.1	74.9
T1	51.9	47.6	49.8
T2	53.3	43.6	48.5
T3	45.9	41.1	43.5
T4	44.3	44.3	44.3
T5	56.1	42.4	49.3
T6	55.3	40.6	47.9
T7	47.1	42.7	44.9

Table 3.
DNA concentration in the BMP 15 and GDF 9 maturation and vitrification.

Groups	DNA concentration of sample group (ng/ µl)	
	BMP-15	GDF-9
(C-) IVM without vitrification	66.89 [a] ± 7.35	73.26 [a] ± 13.63
(T) IVM with vitrification	50.56 [b] ± 4.39	43.19 [b] ± 2.16

Note: Differences in superscript letters in different columns indicate significant differences ($p < 0.05$).

In the negative control group (C-) subjected to in vitro maturation (IVM) without vitrification, the BMP-15 sample group demonstrated a mean DNA concentration of 66.89 ng/µL ± 7.35, whereas the GDF-9 sample group reported 73.26 ng/µL ± 13.63. In the treatment group (P), which received both IVM and vitrification, the DNA concentration diminished to 50.56 ng/µL ± 4.39 in the BMP-15 sample group and to 43.19 ng/µL ± 2.16 in the GDF-9 sample group. Marked differences between the treatment groups ($P<0.05$), indicated by distinct letters in the columns, demonstrate that vitrification significantly lowers DNA concentration in each sample group.

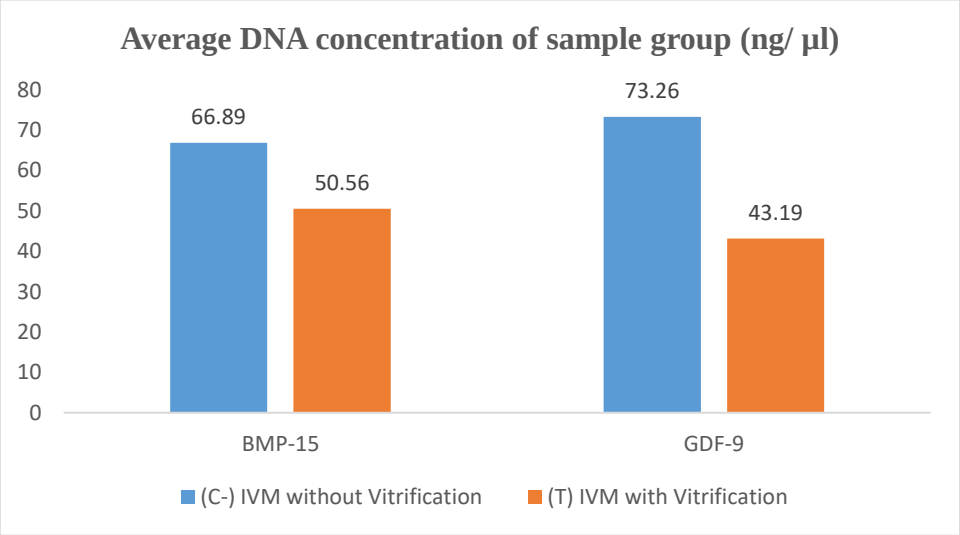

Figure 2.
Graph of the average DNA concentration of BMP 15 and GDF 9 sample groups between the oocyte group after in vitro maturation and the oocyte group after in vitro maturation and vitrification.

Subsequent to these initial observations, the target genes BMP-15 and GDF-9 will undergo analysis by qPCR to enhance specificity.

Table 4.
qPCR amplification of BMP-15 and GDF-9 genes in sheep oocytes.

Sample code	Ct value	
	BMP-15	GDF-9
C-1	31.35	35.93
C -2	25.26	36.89
C -3	Undetermined	37.42
C -4	34.43	Undetermined
C -5	32.55	28.98
C -6	Undetermined	35.39
C -7	34.63	32.34
T1	Undetermined	Undetermined
T2	Undetermined	Undetermined
T3	Undetermined	Undetermined
T4	Undetermined	Undetermined
T5	Undetermined	Undetermined
T6	Undetermined	Undetermined
T7	Undetermined	Undetermined

Table 4 displays the cycle threshold (CT) values for the BMP-15 gene: sample C-1 has a CT of 31.35, C-2 has 25.26, C-4 has 34.43, C-5 has 32.55, and C-7 has 34.63. In contrast, samples C-3 and C-6 had undetermined CT readings. In samples designated T1 to T7, the CT readings were likewise undetermined. An undetermined CT result often denotes a CT value over 40 cycles, indicating little or undetectable expression of the target gene. The CT value indicates the cycle number at which fluorescence exceeds the threshold, thereby confirming the presence of the target nucleic acid. Lower CT values indicate a

higher abundance of the target sequence, whereas higher CT values signal a diminished gene amount. In the case of the GDF-9 gene, sample C-1 recorded a CT value of 35.93, C-2 recorded 36.89, C-3 recorded 37.42, C-5 recorded 28.98, C-6 recorded 35.39, and C-7 recorded 32.34, while sample C-4 remained undetermined. In samples T1 through T7, CT results were undetermined. As a result, in all group P samples, CT values over 40 signify that the GDF-9 gene expression level in group P was inferior compared to group K.

4. Discussion

Establishing DNA concentration is essential for determining the lowest needed concentration for qPCR or real-time PCR processes, as the expression levels of target genes are significantly affected by the quantity of DNA or RNA utilized. Dye-based real-time PCR techniques, such as SYBR Green, enable real-time fluorescence detection by selectively attaching to double-stranded DNA (dsDNA). This dye generates minimal background fluorescence, which markedly increases upon binding to dsDNA, enabling fluorescence levels to grow proportionately with dsDNA synthesis during PCR cycles. This method, requiring just two sequence-specific primers, facilitates swift and economical processing of extensive sample quantities. Nonetheless, a limitation of this dye-based approach is its ability to identify all dsDNA, including non-specific products or primer dimers, which may result in quantification errors. Consequently, a denaturation curve is usually produced following qPCR to confirm reaction specificity and ascertain that only the intended target sequence is amplified.

This study indicates that vitrified oocytes in the treatment group had reduced DNA concentrations relative to the control group, resulting in elevated Ct values, which were frequently indeterminate due to inadequate DNA material for amplification [25]. Furthermore, DNA damage in oocytes during vitrification may potentially arise from the generation of reactive oxygen species (ROS). H_2O_2 is specifically associated with inducing DNA strand breaks through its conversion to hydroxyl radicals, serving as a DNA damage inducer [26].

The GDF-9 and BMP-15 genes are essential for oocyte development in several species, including humans, rodents, ruminants, and marsupials. The GDF-9 gene is expressed from the initial embryonic stage until ovulation [27], and both genes affect granulosa and cumulus cell activities, thereby influencing folliculogenesis, oocyte maturation, and ovulation [28]. Vitrification preserves DNA integrity in follicular cells, as genes encoding GDF-9, BMP-15, TGFBR1, and BPR2 are highly expressed in cumulus-oocyte complexes of both vitrified and non-vitrified ovine oocytes. Nonetheless, GDF-9 gene expression declines in the granulosa cells of immature oocytes [29].

In the in vitro maturation (IVM) process, the concentration of growth factors released into the media is increased [18]. The BMP-15 gene facilitates proper follicle growth, modulates ovulation rates, and inhibits cumulus cell death by sustaining a localized apoptotic gradient among cumulus-oocyte complexes. The vitrification process, however, diminishes the expression of GDF-9 and BMP-15 genes [19]. Cryopreservation, especially of immature oocytes, exhibits differential sensitivity based on the meiotic stage, with immature oocytes being optimal for cryopreservation due to the lack of meiosis [30]. However, immature oocytes exhibit greater susceptibility to osmotic stress and demonstrate reduced membrane stability compared to MII (Meiosis II) stage oocytes. The efficacy of cryopreserving immature oocytes depends on preserving the structural and functional integrity of both the oocyte and its cumulus cells. The timing of gene expression during oocyte maturation is crucial, as the production of GDF-9 and BMP-15 can suppress apoptosis and facilitate oocyte maturation [31].

Vitrification might result in oxidative stress, contributing to mitochondrial dysfunction that profoundly affects biological activities. Increased oxidative stress enhances free radical generation, which may harm DNA and disrupt RNA synthesis, hence diminishing the expression of critical genes, including GDF-9 and BMP-15 [20]. Mitochondrial dysfunction also impairs ATP synthesis in oocytes, hindering the transcription and translation of genes essential for oocyte maturation [32]. The rapid freezing and thawing during vitrification cause abrupt osmotic pressure variations, potentially harming cellular microstructures such as the plasma membrane and cytoskeleton, which are crucial for preserving cellular integrity and gene expression [33]. This structural damage may hinder the signaling pathways essential for maintaining GDF-9 and BMP-15 production during oocyte development.

GDF-9 and BMP-15 depend on autocrine signaling processes facilitated by specific receptors in the oocyte. Vitrification can interfere with these pathways by modifying the activity of enzymes or proteins involved in phosphorylation and dephosphorylation, which are crucial for the activation of autocrine signaling. Disruptions in these pathways diminish the expression of genes essential for maturation, thereby undermining oocyte developmental potential [34].

5. Conclusion

This work illustrates that vitrification negatively impacts BMP-15 and GDF-9 gene expression in ovine oocytes, likely attributable to oxidative stress and structural damage sustained during cryopreservation. Decreased DNA content and elevated Ct values in vitrified oocytes indicate diminished viability and gene expression, adversely affecting oocyte maturation. Consequently, the enhancement of vitrification methods is vital for maintaining oocyte integrity and genetic viability, which is crucial for improving reproductive efficiency and conserving genetic resources in cattle management. We advise augmenting the number of oocytes utilized during extraction for RNA isolation to secure an adequate quantity of DNA for amplification. This method will improve result clarity and enable reliable processing and analysis.

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