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Detection of the *Zygote arrest 1* Gene in Oocytes, Zygotes, and Embryos of Pesisir Cattle with the Addition of IGF-1 within the *In Vitro* Maturation Media

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ABSTRACT

Zygote arrest 1 (*ZAR 1*) is a maternal gene that plays a crucial role in the oocyte-to-embryo transition. The present study aimed to investigate the presence or absence of the *ZAR 1* gene in oocytes, zygotes, and embryos of Pesisir Cattle. Ovaries were collected from cattle at slaughterhouses, and oocytes were retrieved at the Biotechnology Laboratory. The collected oocytes were matured in a maturation medium supplemented with Insulin-like Growth Factor-1 (IGF-1) at a concentration of 10 µg/ml or without IGF-1 for 24 hours in a CO_2 incubator maintained at 38.5°C. Following maturation, the oocytes were fertilized for 18 hours, and the resulting embryos were cultured for 48 hours in a CO_2 incubator at 38.5°C. The samples were then subjected to PCR analysis. The amplification results revealed the presence of the *ZAR 1* gene band at the target size of 228 bp in oocytes matured with and without IGF-1. A comparative analysis of oocytes and embryos showed differences in the gene bands, particularly in samples supplemented with IGF-1. These findings suggest that IGF-1 supplementation during oocyte maturation significantly influences *ZAR 1* gene expression in embryos. The observed variations in *ZAR 1* gene expression across the oocyte, zygote, and embryo stages highlight the gene's pivotal role in reprogramming post-fertilization and maintaining early embryonic development.

Keywords: Embryo, Insulin-like Growth Factor-1, Oocyte, Zygote, *Zygote arrest 1* gene

INTRODUCTION

Pesisir cattle are a local breed from West Sumatra with significant potential as meat producers. They demonstrate strong adaptability to coastal environments, even in regions with limited forage availability. This adaptability offers opportunities to expand their development across the coastal areas of Indonesia. However, the primary challenges in developing Pesisir cattle include low productivity and declining genetic quality. Genetic improvement efforts have been implemented to enhance the productivity of Pesisir cattle (Adrial, 2010). One approach to genetic improvement involves the application of reproductive technologies (Suryana, 2009). The ovary, a vital reproductive organ, produces fertilized oocytes with optimal developmental potential and secretes steroid hormones necessary for preparing the reproductive tract for fertilization and implantation (Palermo, 2007). Dysfunction in the ovary can disrupt the reproductive system, and numerous studies have explored using bioactive compounds to enhance reproductive performance (Abdullah et al., 2018). One such bioactive compound is Insulin-like Growth Factor-1 (IGF-1), which plays a crucial role in the female reproductive system. IGF-1 regulates growth, cell differentiation, cell metabolism, and apoptosis, contributing significantly to early embryonic development (Byrne et al., 2002).

According to Abdullah et al. (2018), the supplementation of Insulin-like Growth Factor-1 (IGF-1) in oocyte maturation and culture media stimulates and enhances oocyte maturation, improves *in vitro* fertilization (IVF) outcomes, and increases the number of embryos reaching the blastocyst stage in various livestock species, including pigs and cattle (Neira et al., 2010) as well as buffaloes (Singhal et al., 2009). In livestock, particularly cattle, IVF is one approach to utilizing ovarian waste from female cows slaughtered in abattoirs. The process of IVF is expected to produce cattle embryos that can be transferred to recipient cows, thereby enhancing reproductive efficiency and genetic improvement efforts (Kaiin et al., 2005).

The development of an embryo through fertilization, cleavage, blastocyst formation, and implantation are highly dependent on the quality of the oocytes. The role of the oocyte is particularly critical during the maternal-embryonic transition (MET), the interval between fertilization and the activation of the embryonic genome's transcriptional activity.

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During this period, embryonic development is supported by maternal RNA and proteins synthesized during oogenesis (Telford et al., 1990; Brevini et al., 2004). The duration of the MET varies among species. In mammals, this process occurs at different developmental stages depending on the species (Brevini et al., 2004). In mice, MET occurs at the late 2-cell stage, while in pigs, it occurs at the 4-cell stage. In human embryos, it happens between the 4-cell and 8-cell stages, in rabbits at the 8-cell stage, and in sheep and cattle embryos between the 9-cell and 16-cell stages (Telford et al., 1990; Brevini et al., 2004).

The quest for enhanced reproductive efficiency in cattle has increasingly focused on optimizing *in vitro* fertilization (IVF) techniques. A pivotal factor in IVF's success is oocyte maturation, which directly influences embryo quality (Yang et al., 2022). Recent studies have highlighted the significant role of Insulin-like Growth Factor-1 (IGF-1) in promoting the developmental competence of bovine oocytes (Spicer et al., 2002). IGF-1 facilitates various cellular processes, including proliferation, differentiation, and metabolic regulation, improving maturation outcomes (Spicer et al., 2002; Yang et al., 2022).

One of the techniques for assessing the success of pre-implantation development in animals is highly dependent on maternal RNA and proteins synthesized during oogenesis in the early stages of embryo development. An important oocyte-specific maternal effect gene for early embryo development is *Zygote arrest 1* (*ZAR 1*, Brevini et al., 2004). *Zygote arrest 1* is a maternal gene that plays a crucial role in the oocyte-embryo transition, potentially providing new insights into the initiation of embryonic development and the control of mammal fertility. The *ZAR 1* gene is evolutionarily conserved in vertebrates, and the *ZAR 1* protein is characterized by the presence of an atypical plant homeobox zinc finger domain, highlighting its role in transcriptional regulation. Furthermore, the *ZAR 1* gene is considered one of the key regulators of successful pre-implantation development in pigs and cattle (Brevini et al., 2004; Uzbekova et al., 2006).

The *ZAR 1* gene is expressed in oocytes, zygotes, and all stages of embryonic development up to blastocyst formation. Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis was performed on oocytes and pre-implantation embryos produced *in vitro* (Brevini et al., 2004). Brevini et al. (2004) stated that the *ZAR 1* gene is the most recently identified maternal effect gene in mice and is also the most specific. The mRNA of the *ZAR 1* gene is present in oocytes and 1-cell embryos, with a significant decline observed at the 2-cell stage. In cattle, however, the *ZAR 1* gene is expressed across all stages of cell division but shows a decrease after the 2-cell stage. Meanwhile, studies by Pennetier et al. (2005) and Uzbekova et al. (2006) indicate that the *ZAR 1* gene is present in oocytes and 4-cell embryos but decreases notably at the 8-cell stage.

The *ZAR 1* gene is crucial for regulating the timing of early cell divisions during embryogenesis. Dysregulation of *ZAR 1* gene expression can lead to *Zygote arrest*, negatively impacting embryo viability and overall reproductive success (Michailidis et al., 2009). Thus, understanding how IGF-1 influences *ZAR 1* gene expression during oocyte maturation and subsequent embryonic development is essential for refining *in vitro* maturation (IVM) protocols. Recent findings indicate that adding IGF-1 to the maturation medium enhances oocyte quality and alters the expression of essential genes associated with embryo development, including the *ZAR 1* gene (Brevini et al., 2004).

Gene expression studies during embryogenesis in cattle are still limited, as they have only been carried out in a single species. Therefore, they are inadequate and need to be further elucidated to provide a model for controlling gene expression during early development in mammals, as well as zygotic or embryonic gene activation in cattle. A detailed analysis of gene expression during cattle embryogenesis is essential for understanding the basic cellular and molecular mechanisms of gene expression control, developing better embryo culture systems, and improving strategies for transgenic and cloning studies. This gene expression analysis method can be valuable for researchers engaged in animal and livestock genetics at the cellular and molecular levels. Insights into oocyte maturation with the addition of IGF-1 inform strategies to improve embryo quality and reproductive outcomes in cattle, contributing to advancing reproductive technologies in livestock production. The present study aimed to determine the presence or absence of the *ZAR 1* gene in oocytes, zygotes, and embryos of Pesisir Cattle.

MATERIALS AND METHODS

Materials

The Pesisir cattle are native to the coastal areas of West Sumatra. 16 pairs of ovaries from Pesisir Cattle were obtained from the Padang slaughterhouse (RPH). At the same time, semen samples were sourced from Friesian Holstein (FH) bulls produced by the Artificial Insemination Center in Lembang, Indonesia.

The materials used included NaCl, penicillin (100 IU/ml, Meiji, Japan), streptomycin (0.1 µg/ml, Meiji, Japan), Tissue Culture Medium (TCM)-199 (Sigma, M4530, UK), Pregnant Mare Serum Gonadotropin (PMSG), Insulin-like Growth Factor-1 (IGF-1, Sigma, UK), Bovine Serum Albumin (BSA, 0.3%, Sigma, A2153, UK), gentamicin (50 µg/ml,

Sigma, G1397, UK), Brackett-Oliphant (BO) medium, PBS (phosphate-buffered saline) solution, Aquabides, and mineral oil (Sigma, USA), 2% agarose, primers, master mix, Taq DNA polymerase, and 1-TAE buffer. The tools used included a stereo microscope (Nikon, Japan).

Methods

Collecting ovaries and oocytes

The procedure was carried out according to Nanda et al. (2019). The cattle ovaries obtained from the slaughterhouse were transported to the laboratory in a physiological NaCl medium supplemented with 100 µg/ml streptomycin (Meiji, Japan) and 100 IU/ml penicillin (Meiji, Japan) and stored in a thermos at 30-35°C. The ovaries were then thoroughly rinsed, and the oocytes were collected no later than 6 to 8 hours after incision. The collection of oocytes from the ovaries was performed by making incisions at surgical sites on the ovaries. The ovaries were placed in a petri dish containing 5 ml of collection medium, including PBS solution, and held using tweezers. Follicles on the surface of the ovaries were then incised using a scalpel. The follicular fluid and the PBS solution caused the oocytes to be released and collected. The oocytes in the petri dish were then selected under a stereomicroscope.

The selected oocytes were evaluated for quality and classified based on morphology according to the criteria described in previous studies (de Loos et al., 1989; Blondin and Sirard, 1995; Yuan et al., 2005). G1 represented the highest quality oocytes, characterized by five or more layers of compact cumulus cells. G2 included good-quality oocytes, identified by a uniform structure and fewer than five layers of cumulus cells. G3 oocytes were identified by scattered cumulus cells. The oocytes used in the current study were classified as grades A, B, and C.

The selected oocytes were then matured using TCM-199 medium, 10 µg/ml PMSG, BSA, without IGF-1, 10 µg/ml IGF-1 (Sigma, 1-5500), and 50 µg/ml gentamicin. The selected oocytes were rinsed thrice and then matured in a petri dish for 24 hours in a 5% CO_2 incubator at 38.5°C. The oocytes were cultured in 100 µL drops containing 10-15 oocytes and covered with mineral oil (Sigma-Aldrich, Inc., M-8410). All media used in the present study were equilibrated for at least 2 hours in a 5% CO_2 incubator at 38.5°C before use. After the oocytes had matured for 24 hours, two groups of oocytes were taken to observe *ZAR 1* gene expression by PCR, and the remaining oocytes were used for the *in vitro* fertilization stage.

In vitro fertilization

The procedure was carried out according to Nanda et al. (2019). Oocyte fertilization was performed using frozen semen from FH cattle. The frozen semen was thawed in warm water at 30-37°C for 30 seconds, then placed into a sterile tube and centrifuged at 700 g for 8 minutes. After centrifugation, the supernatant was removed, and the spermatozoa were diluted with BO medium to achieve a final concentration of 2×10^6 spermatozoa/mL. The spermatozoa and BO medium mixture were made into 100 µL petri dish drops for 10-15 oocytes, covered with mineral oil (Sigma, USA). The matured oocytes were washed twice in BO medium, then transferred to the respective drop according to treatment, and incubated in a 5% CO_2 incubator at 38.5°C for 18 hours (Nanda et al., 2019). After 18 hours of fertilization, two groups of fertilized oocytes were taken to observe *ZAR 1* gene expression by PCR, while the remaining fertilized oocytes were observed for *ZAR 1* gene expression in embryos after 48 hours of fertilization.

Zygote arrest 1 gene amplification

Polymerase chain reaction (PCR) was performed with cDNA equivalents corresponding to at least two batches of oocytes, zygotes, or embryos. The master mix reaction mixture consisted of 1x $MgCl_2$, 25 U Taq DNA polymerase, 1x PCR buffer (Tris-HCl, KCl), 2.5 mM dNTPs, primers, and sterile water.

Zygote arrest 1 amplification was performed in an automatic thermal cycler using the following conditions, including 2 minutes at 94°C (denaturation temperature), 10 seconds at 60°C (annealing temperature), and 2 minutes at 72°C (extension temperature) for 35 cycles. The primers were L-ACGTCGTCCTGGATGGTTAC and R-GCTGGTAGCTGTGGACGTACT, which encoded a 228 bp product.

PCR products were electrophoresed on a standard 2% agarose gel in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) with a total volume of 350 mL, using a 9 mm gel tray. Electrophoresis was performed at 80 V for 45 minutes, after which the fragments were visualized using a UV transilluminator at 312 nm. Each gel was then documented with a camera (Brevini et al., 2004; Uzbekova et al., 2006).

Variables

Determining whether there are differences in *ZAR 1* gene expression in oocytes, zygotes, and embryos of Pesisir cattle with and without IGF-1 treatment.

Statistical analysis

The data obtained from the current study are qualitative, in the form of band images of *ZAR 1* gene expression in oocytes, zygotes, and embryos of Pesisir cattle with and without IGF-1 treatment during maturation. For descriptive data, frequency and percentage were used to visualize the results. Data analysis was conducted using the Chi-Square test, with a p-value of less than 0.05, which was considered statistically significant. The analyses were performed using SPSS Statistics version 25.

RESULTS AND DISCUSSION

The influence of cumulus cells on the *ZAR 1* gene

Oocytes with cumulus cells used in the present study (Table 1) were grouped into three categories.

From Table 1, oocytes supplemented with IGF-1 during the maturation medium exhibited more significant cumulus cell swelling than those without IGF-1. According to Da Broi et al. (2018), cumulus cells support oocyte maturation through metabolic substances. Cumulus cells are crucial in providing nutrients to the oocyte and assisting in protein synthesis for zona pellucida formation. *In vitro*, oocyte maturation is a process influenced by individual oocyte characteristics, stemming from differences in the development and growth of oocytes collected from the ovaries (Conti and Franciosi, 2018).

During *in vitro oocyte* maturation, changes in cumulus cell morphology and interactions between follicular cells and the oocyte occur. Cumulus cells are essential for enhancing cytoplasmic maturation, which is necessary for pronucleus formation and the ability to continue development (Muhajir, 2018). Oocytes with cumulus cells have a higher fertilization capacity compared to those that mature without them. This is attributed to cumulus cells reducing the degree of *zona pellucida* hardening during culture. Hardening of the zona pellucida decreases the fertilization capacity of oocytes matured without cumulus cells (Kusindarta, 2009). Sato et al. (2018) demonstrated that insulin promoted oocyte maturation in cattle and the swelling of cumulus cells, and it could inhibit apoptosis. Insulin also enhanced *in vitro* oocyte maturation, fertilization, and embryonic development to the blastocyst stage in mice (Kiapekou et al., 2005). Adding insulin to the IVM medium improved pig models' maturation rates and increased IVF outcomes (Xia et al., 1994). Cumulus cells play a vital role in oocyte maturation and fertilization by releasing and mediating signals to the oocyte (Auclair et al., 2013).

Table 1. Matured results of oocytes from Pesisir cattle were used for PCR

Treatment	Amplification	Cumulus cell		
		G1	G2	G3
A (without IGF-1)	1	-	3	12
	2	-	9	6
	3	3	10	2
	4	5	10	-
B (IGF-1 10 µg/ml)	1	-	10	5
	2	4	7	4
	3	7	8	-
	4	10	5	-

Note: G1: oocyte lined with > 5 layers of compact cumulus cells, G2: With only 1-5 layers of compact cumulus cells, G3: With scattered cumulus cells.

Detection of the *Zygote arrest 1* gene

Detection of the *ZAR 1* gene using primer pairs L ACGTCGTCCTGGATGGTTAC and R-GCTGGTAGCTGTGGACGTACT. As many as 120 samples were amplified in Table 2, and the amplification can be seen in Figure 1.

The amplified samples (Table 2) without IGF-1 showed the presence of the *ZAR 1* gene in 50% of oocytes, 75% of zygotes, and 25% of embryos. In contrast, with the addition of 10 µg/ml IGF-1, the *ZAR 1* gene was detected in 75% of oocytes, zygotes, and embryos. These results indicated that adding IGF-1 to the maturation medium provides more significant benefits than its absence. However, the results showed no significant effect between the addition of IGF-1 and its absence on the *ZAR 1* gene in oocytes and zygotes ($p > 0.05$), although the addition of IGF-1 had a significant effect on *ZAR 1* gene detection in Pesisir cattle embryos ($p < 0.05$).

According to Bonilla et al. (2011), preimplantation embryo conditions depended on the endocrine environment that regulated its developmental program. Growth factors such as insulin were one endocrine signal influencing the embryo's

ability to develop in optimal and suboptimal environments. In cattle, insulin increased the proportion of preimplantation embryos developing to the blastocyst stage, altered blastocyst gene expression, enhanced the resilience of preimplantation embryos on days 4-6 against heat shock, and improved embryo survival after transfer to recipients. Boucher et al. (2014) noted that insulin and IGF-I regulated different biological processes, such as cell metabolism, proliferation, differentiation, and apoptosis, which are mediated by activating intrinsic tyrosine kinase activity at their receptors. The loss of insulin and IGF-I receptors in cells significantly decreased the expression of several genes, both maternally and paternally expressed.

According to Sánchez et al. (2009) and Wasielak et al. (2016), exogenous growth factors like insulin *in vitro* influenced the RNA of developmental genes, including the *ZAR 1* gene, which played a role during the early stages of embryonic development. Racedo et al. (2009) reported that *ZAR 1* mRNA levels were influenced by maturation stages in isolated oocytes, showing a decrease during the maturation of oocytes isolated from larger follicles while remaining relatively stable in those from smaller follicles.

Table 2. Amplification results of oocytes, zygotes, and embryos from Pesisir cattle

Treatment	Samples	Early development of embryo (%)			P-value
		Oocyte (20 samples)	Zygote (20 samples)	Embryo (20 samples)	
A	60	($^{10}/_{20}$) 50%	($^{15}/_{20}$) 75%	($^{5}/_{20}$) 25%	
B	60	($^{15}/_{20}$) 75%	($^{15}/_{20}$) 75%	($^{15}/_{20}$) 75%*	$p < 0.05$

Note: A: Without IGf-1, B: IGF-110 µg/ml, n: Sample size, the numbers in parentheses indicate the number of samples.* indicates a significant difference in a column.

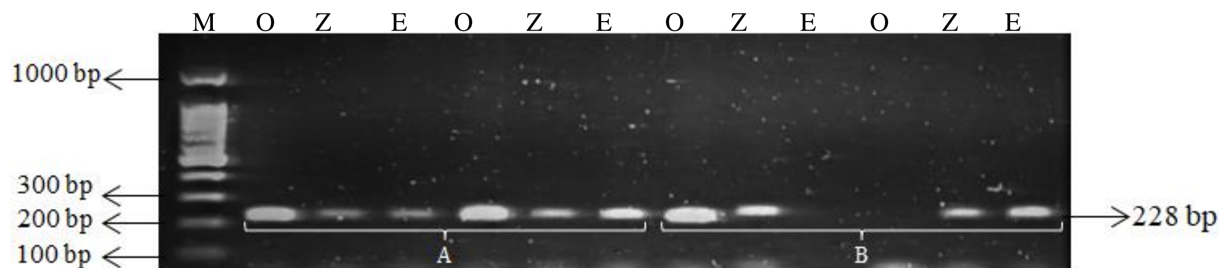

Figure 1. ZAR gene detection 1. **A:** IGF-1 addition to the maturation media, **B:** Without addition of IGF-1 to the maturation media. M (Marker), O (Oocytes), Z (Zygote), E (Embryo).

The *ZAR 1* gene was amplified explicitly because only one DNA band in each sample was electrophoresed according to the expected size of 228 bp (Figure 1). The amplification results from Figure 1A indicated that samples matured with the addition of IGF-1 showed a band for the *ZAR 1* gene in oocytes, zygotes, and embryos. In contrast, the amplification results from Figure 1B showed a difference in samples without adding IGF-1, as there was no *ZAR 1* gene band in oocytes and embryos, although a band for the *ZAR 1* gene was present in the zygotes.

The detection of the *ZAR 1* gene in Pesisir cattle embryos showed differences compared to the *ZAR 1* gene in FH cattle. In the study by Brevini et al. (2004), *ZAR 1* was detected up to the blastocyst stage with an increase in mRNA at the four-cell stage. Wasielak et al. (2016) indicated differences in *ZAR 1* mRNA levels produced by *in vivo* and *in vitro* embryos. *In vivo*, *ZAR 1* mRNA decreased starting from the four-cell stage, whereas *in vitro*, the decrease occurred earlier at the two-cell stage. The *ZAR 1* protein participated in interactions related to chromatin-mediated transcription regulation during the oocyte-embryo transition.

As noted in the previous study by Shirazi and Motaghi (2013), *ZAR 1* was a maternal-specific ovarian factor that played a crucial role during pregnancy. The transition from oocyte to embryo in mice showed that its expression was highly restricted to oocytes, zygotes, and, to a lesser extent, two-cell embryos. In *ZAR 1* knockout mice, embryos failed to develop beyond the first cleavage stage. Other studies found that less than 20% of embryos from *ZAR 1* (-/-) females progressed to the two-cell stage, showing a significant reduction in the synthesis of complexes requiring transcription, with no embryos developing to the four-cell stage (Wu et al., 2003a; Shirazi and Motaghi, 2013). According to the study by Brevini et al. (2004), the *ZAR 1* gene was observed up to the 8-cell stage.

Maternal genes were expressed in oocytes and embryos and played a crucial role in activating the embryonic genome. Abnormalities in the expression of these genes could lead to halted embryonic cleavage or transcriptional changes responsible for further embryonic development (Wasielak et al., 2016). The *ZAR 1* gene was important during the first stages of embryonic development, and the absence of *ZAR 1* protein in oocytes directly impacted cleavage potential and the development of embryos beyond the blastocyst stage (Sánchez et al., 2009). These results indicated that

maternal genes played a significant role in reprogramming after fertilization or maintenance during early pre-implantation embryos (Tsunemoto et al., 2008; Racedo et al., 2009).

The role of the *ZAR 1* gene in early embryonic development was essential, particularly in the context of bovine reproduction (Wasielak et al., 2016). Investigation into the expression of *ZAR 1* in oocytes, zygotes, and embryos in the presence of IGF-1 during *in vitro* maturation (IVM) revealed significant insights into its regulatory mechanisms and potential applications in enhancing reproductive efficiency (Brevini et al., 2004).

According to Yang et al. (2022), IGF-1 supplementation during *in vitro* maturation significantly increased the maturation rates of bovine oocytes, which was essential for successful fertilization and subsequent embryonic development. This improvement was attributed to IGF-1's role in promoting metabolic activity and enhancing the physiological conditions necessary for oocyte maturation. In the findings, *ZAR 1* expression was notably elevated in oocytes matured with IGF-1, supporting the hypothesis that this growth factor played a vital role in the transcriptional regulation of genes critical for early embryogenesis (Spicer et al., 2002).

The expression patterns of *ZAR 1* during the zygotic stage also warranted attention. Analysis indicated that *ZAR 1* remained expressed post-fertilization, which aligned with findings highlighting its importance in regulating the timing of embryonic cleavage divisions. Adding IGF-1 appeared to stabilize *ZAR 1* expression in zygotes, potentially facilitating a more robust transition from maternal to embryonic development control (Wu et al., 2003b). This stabilization mitigates the effects of *Zygote arrest*, a common issue that can lead to developmental failures (Wu et al., 2003a).

Furthermore, the impact of IGF-1 extended into the later stages of embryonic development. The presence of IGF-1 enhanced the initial stages of maturation and fertilization and supported progression to the blastocyst stage. The present results indicated that embryos developed with IGF-1 exhibited increased *ZAR 1* expression, correlating with higher blastocyst rates. This finding aligned with previous studies that underscored the role of *ZAR 1* in maintaining embryonic viability (Brevini et al., 2004; Michailidis et al., 2009).

However, it was crucial to recognize that while IGF-1 supplementation appeared beneficial, the timing and concentration of its application required careful optimization. Excessive levels may have altered gene expression patterns and unintended effects on embryonic development (Neira et al., 2010). Thus, further studies are warranted to elucidate the optimal conditions for IGF-1 use during IVM and its long-term implications for embryo quality and reproductive success.

CONCLUSION

The *ZAR 1* gene was detected in oocytes, zygotes, and Pesisir cattle embryos. The study revealed differences in the detection of the *ZAR 1* gene in oocytes, zygotes, and embryos of Pesisir cattle with or without adding IGF-1 in the *in vitro* maturation medium. The presence of the *ZAR 1* gene at the oocyte, zygote, and embryo stages indicated that maternal genes played important roles in reprogramming after fertilization or in maintaining early embryonic development. Based on the study results, further research should concentrate on the effects of IGF-1 supplementation in embryo culture media and detecting the *ZAR 1* gene during cleavage stages, from the four-cell to the blastocyst stage.

DECLARATIONS

Authors' contributions

All authors contributed to the study's conception, design, statistical analysis, and practical duties in the study. The first draft of the manuscript was written by Sedrisa Lidya Pertiwi, and Tinda Afriyani and Jaswandi commented on previous versions of the manuscript and reviewed the study. All authors read and approved the final version of the manuscript.

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Ethical considerations

This paper was originally written by the authors and has not been published elsewhere. The authors checked the text of the article for plagiarism index and confirmed that the text of the article is written based on their original scientific results.

Availability of data and materials

The data to support this study's findings is available upon reasonable request to the corresponding author.

Competing interests

The authors have no competing interests to declare.

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