

Research Article



The Effect of using HEPES in Culture Medium on Oocyte Maturation Rates and *In vitro* Fertilization in Pesisir Cattle

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Abstract | This research aims to assess oocyte maturation rates, in vitro fertilization, and pronuclei development in Pesisir cattle, and to determine the optimal culture medium and culture duration for in vitro fertilization techniques. The culture media used include TCM-199 medium supplemented with both B-O (*Brckett Oliphant*) medium (HEPES, without the use of 5% CO₂) and modified B-O (mB-O) medium (without HEPES, with 5% CO₂). For the oocyte maturation stage, the culture period was 24 hours, while for in vitro fertilization, various culture periods (6, 12, 18h) were utilized. The analysis of research data acquisition was conducted using a Completely Randomized Design, utilizing SPSS. The results of in vitro maturation of Pesisir cattle oocytes indicate the development of GV (*Germinal Vesicle*), GVBD (*Germinal Vesicle Breakdown*), M-I (Maturation-I) and M-II (Maturation-II) cells in different culture media as follows: B-O medium (14.29%, 0%, 33.33%, 57.14%), mB-O (17.65%, 0%, 23.53%, 58.82%). The results of in vitro fertilization rates using different culture media at various incubation periods were as follows: B-O medium at 6, 12, and 18h yields 57.14%, 52.17%, and 48.65%, while mB-O medium yields 57.69%, 51.22%, and 48.28%. The research concludes that the addition of HEPES in the B-O medium can substitute for the role of 5% CO₂ in the mB-O medium and even enhance oocyte maturation and in vitro fertilization in Pesisir cattle. This demonstrates that using HEPES buffer facilitates in vitro embryo production outside the laboratory, making it a more accessible, efficient, and cost-effective option.

Keywords | *Brckett Oliphant*, HEPES, 5% CO₂, Oocyte Maturation, *In vitro* Fertilization, Pronucleus

Received | September 23, 2023; **Accepted** | April 03, 2024; **Published** | September 05, 2024

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Citation | Syaiful FL, Jaswandi J, Mundana M, Udin Z (2024). The effect of using HEPES in culture medium on oocyte maturation rates and *In vitro* fertilization in pesisir cattle. J. Anim. Health Prod. 12(3): 450-457.

DOI | <http://dx.doi.org/10.17582/journal.jahp/2024/12.3.450.457>

ISSN (Online) | 2308-2801



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INTRODUCTION

One of the most pressing challenges within the livestock sector is the livestock's low productivity and declining genetic quality. To combat this issue, the utilization of animal biotechnology techniques, including in vitro fertilization (IVF), has become imperative. IVF technology represents one of the most promising methods for augmenting cattle productivity, encompassing enhancements in oocyte maturation and in vitro fertilization rates aimed at producing high-quality embryos. According to Ball and Peter (2007), IVF technology has the potential to yield

more embryos than natural mating, offering a viable alternative for embryo production. Additionally, as mentioned by Kochhar et al. (2002), IVF technology can be employed to generate in vitro embryos using ovaries procured from slaughterhouses. This aligns with the findings of Loner-gan et al. (2016), which suggest that ovaries obtained from slaughterhouses may exhibit varying qualities. Hence, selecting ovaries and oocytes from superior cattle is imperative to bolster in vitro embryo production.

The pivotal phase in IVF technology ensures the oocyte's development to the maturation stage-II (M-II) and its

subsequent transformation into embryos. As suggested by Rahman et al. (2008), the success of oocyte maturation and in vitro fertilization hinges on selecting the appropriate culture medium and system. To facilitate oocyte and embryo development, opting for a suitable culture medium furnished with the necessary nutrients is imperative. Bahrami et al. (2023) noted that the triumph of in vitro cattle embryo production is intricately tied to oocyte maturation, with TCM-199 medium typically yielding high maturation rates. Furthermore, Nedambale et al. (2006) observe that *Brackett Oliphant* (B-O) medium is a prevalent type of cell culture medium employed in in vitro fertilization (IVF) and embryo development. B-O medium, being a straightforward buffered medium, is often employed in combination with other media like modified medium 199 (IVF-M199) or Tyrode's albumin lactate pyruvate (TALP) to optimize IVF and embryo development rates, as elucidated by Khurchabilig et al. (2020), O'Shea et al. (2017), and Assidi et al. (2011). Selecting appropriate nutrient substances tailored to cell culture requirements can bolster oocyte maturation, cumulus cell development, and the transformation of oocytes into embryos.

Conversely, Will et al. (2011) emphasize that one of the factors impacting the success of in vitro embryo culture is pH. Inadequate pH levels in cell culture can induce stress in cells and embryos, potentially resulting in cell or embryo mortality. Hence, maintaining pH stability within the culture medium is paramount, as pH plays a pivotal role in refining the cell and embryo culture processes. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) is one of the most commonly employed buffers in cell culture media for pH maintenance. According to Putra (2019), HEPES stands out as a zwitterionic buffer, distinguished by its remarkable purity level (99.5%) and ability to steadfastly uphold the physiological pH in cell cultures. HEPES also serves the purpose of pH stabilization within the culture medium. It is frequently utilized as a substitute for bicarbonate buffering in cell culture media due to its resilience in fluctuating CO₂ concentrations, eliminating the need for a CO₂ incubator. The paramount role played by HEPES in vitro cell culture lies in its capability to ensure and sustain medium pH, both during storage and cultivation. Jaswandi (2002) elaborated that adding HEPES buffer to the culture medium can elevate in vitro sheep fertilization rates to as high as 57.16%.

Appreciating the benefits of the HEPES buffer, the current study developed an interest in investigating the efficacy of supplementing HEPES in various culture media to substitute the need for 5% CO₂. This approach offers the potential for conducting in vitro embryo production beyond the confines of the laboratory, with the expectation of greater efficiency, cost-effectiveness, and enhanced feasibility for future in vitro embryo production endeavors.

The research aims to assess oocyte maturation rates, in vitro fertilization, the progression of pronucleus development in Pesisir cattle, and determine the most suitable culture medium and duration for in vitro fertilization techniques.

MATERIALS AND METHODS

RESEARCH MATERIALS

The research materials comprised ovaries from Pesisir cows acquired from a slaughterhouse in Padang City, West Sumatra, Indonesia. Fresh semen, utilized for in vitro fertilization, was procured from FH (Frisian Holstein) bulls. This research was conducted at the Laboratory of Animal Biotechnology, Faculty of Animal Science, Universitas Andalas, West Sumatra-Indonesia.

ETHICAL APPROVAL

The experimental research related to animals was evaluated and approved by the research ethics committee at the Faculty of Medicine of Universitas Andalas, West Sumatra-Indonesia.

RESEARCH METHOD

This study involves two treatments, each replicated ten times. Each replication consists of 30-50 oocytes. The treatments are as follows: Treatment 1 (T1): B-O medium (HEPES), and Treatment 2 (T2): Modified B-O medium (mB-O (without HEPES)). The research process encompasses two stages: (1) In vitro oocyte maturation and (2) in vitro fertilization.

OVARIAN COLLECTION

The ovaries were sourced from female Pesisir cows that were processed at the Animal Slaughterhouse in Padang City, West Sumatra. These ovaries were then immersed in a specially prepared medium of 0.9% physiological NaCl solution enhanced with 100 IU/mL of streptomycin. Subsequently, the collected ovaries were transported to the laboratory inside an ovarian collection thermos, maintaining a temperature between 35-37°C, all within a maximum of 2h post-slaughter, according to Parera and Hadisutanto (2014). The ovaries underwent three rinses in the laboratory using a 0.9% physiological NaCl solution. For oocyte retrieval, a collection medium was formulated by employing phosphate-buffered saline (PBS) enriched with 10% bovine serum and gentamicin at a concentration of 50µg/mL. Oocyte retrieval was carried out by sectioning the acquired cow ovaries, as described by Novitasari et al. (2022) and Wang et al. (2007)

IN VITRO OOCYTE MATURATION

The collected oocytes were initially scrutinized under a microscope, and those of quality A and B were selected. Subsequently, the chosen oocytes were placed in Petri dishes

for a thorough washing process, employing a PBS medium. During the oocyte maturation phase, two types of media were employed: *Brackett Oliphant* (B-O) medium and modified B-O (mB-O) medium. [Honkawa et al. \(2018\)](#) prescribed that the oocytes be cultivated in the maturation medium (30-50 oocytes per 100µL droplet) under a layer of mineral oil. The B-O maturation medium consisted of TCM-199 medium supplemented with HEPES at 20 mM, 10% fetal bovine serum (FBS), 10 µg/mL FSH (follicle stimulating hormone), and 50µg/mL gentamicin. The in vitro maturation process was conducted without 5% CO₂ at a temperature of 38.5°C. In contrast, the modified B-O (mB-O) treatment omitted HEPES and was composed of TCM-199 medium, 10% FBS, 10 µg/mL FSH, and 50µg/mL gentamicin. Oocyte maturation took place at 38.5°C with 5% CO₂ for a duration of 24h, in accordance with [Hasbi et al. \(2017\)](#).

Oocyte maturation assessment was carried out following an adapted procedure ([Vodkova et al., 2008](#); [Davachi et al., 2014](#)). Initially, matured oocytes were subjected to a PBS wash to eliminate adhering cumulus cells. Subsequently, the oocytes were fixed by immersion in a solution containing acetic acid and absolute ethanol in a 1:3 ratio and were left to soak for 48h. Following this, the oocytes were stained with a 1% aceto-orcein solution (Sigma, O-7380) for 10 minutes, and their developmental status was observed using a microscope. [Gordon \(2003\)](#) proposed that mature oocytes are identifiable by cumulus cell expansion and the formation of polar body-1 (PB-1).

IN VITRO FERTILIZATION

The in vitro fertilization technique followed the procedures of [Imai et al. \(2006\)](#), [Brackett and Oliphant \(1975\)](#), and [Sugimura et al. \(2010\)](#) with modifications. In vitro fertilization induced capacitation by incubating the spermatozoa in the treatment media, namely B-O and mB-O media. The collected sperm were washed in 4mL of treatment media (B-O and mB-O). They were then loaded into a centrifuge tube containing 200µL of bull sperm and centrifuged at 500 G for 10 minutes. After centrifugation, the resulting supernatant was discarded, and the sedimented sperm were diluted with a culture medium to achieve a concentration of 3x10⁶ spermatozoa/mL. Subsequently, the sperm were incubated for 30 minutes at 38.5°C.

For mature oocytes, a washing process was conducted to remove some cumulus cells using culture media (B-O and mB-O) supplemented with 1N NaOH to achieve a pH of 7.4. The in vitro fertilization began by placing 20-30 mature oocytes into the treatment medium containing 50µL of capacitated sperm. The preparation of the treatment medium followed the oocyte maturation procedure. Subsequently, the fertilized oocytes were incubated at 38.5°C

for 6, 12, 18h. In vitro fertilization was evaluated following the oocyte maturation procedure using aceto-orcein staining. This evaluation observed pronucleus development in 1PN, 2PN, and >2PN.

STATISTICAL ANALYSIS

The collected data comprised oocyte maturation rates, in vitro fertilization, and pronucleus development. The data obtained from the research were analyzed using the Chi-square Test using the SPSS 23.0 version software.

RESULTS AND DISCUSSION

IN VITRO OOCYTE MATURATION

The study findings disclosed the percentage of in vitro maturation (M-II) of Pesisir cattle oocytes in various incubation media over 24 hours, as illustrated in [Figure 1](#).

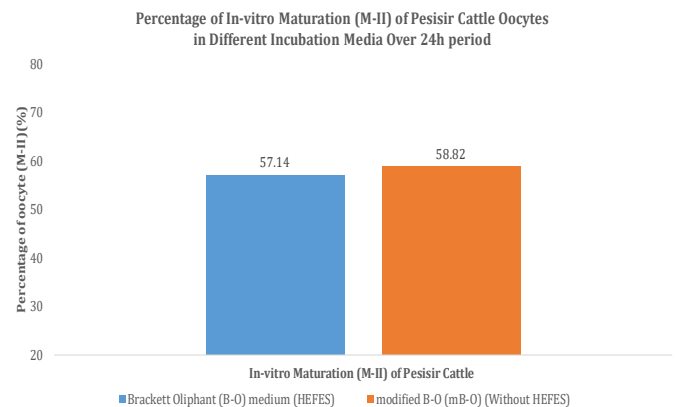


Figure 1: Average percentage of in vitro maturation (M-II) of Pesisir cattle oocytes in different incubation media over a 24-hour period.

[Figure 1](#) shows that the average percentage of in vitro maturation (M-II) in Pesisir cattle oocytes is highest when using the *Brackett Oliphant* (B-O) medium, with a rate of 58.82%. Conversely, the lowest percentage was attained when the modified B-O (mB-O) medium was employed, with a rate of 57.14%. Statistical analysis of the data indicates that the use of different culture media does not have a significant ($P>0.05$) impact on the in vitro maturation rate of Pesisir cattle oocytes (M-II). This can be attributed to the inclusion of HEPES in the B-O medium, which can replace the function of 5% CO₂. Accordance to [Putra \(2019\)](#), incorporating HEPES into culture media helps maintain pH stability. HEPES is frequently employed as a substitute for bicarbonate buffering in cell culture media because it is less sensitive to fluctuations in CO₂ concentration and does not necessitate a CO₂ incubator.

The results of this study align with those of [Barceló-Fimbres et al. \(2015\)](#), who reported that the 24-hour in vitro maturation of cow oocytes without CO₂ gas does not

affect the proportion of immature oocytes reaching the metaphase-II stage. Based on the results of this study, it is clear that HEPES buffer supplementation effectively substitutes for the role of 5% CO₂ in maintaining the pH of the culture medium, thus creating optimal conditions for in vitro oocyte maturation. Including HEPES in the maturation medium provides advantages in the in vitro oocyte maturation process without relying on a 5% CO₂ incubator. This is corroborated by Dode et al. (2002), who found that the embryo development rates from oocyte maturation and IVF without 5% CO₂ were not significantly different from those cultured in a 5% CO₂ incubator.

The cow oocyte maturation process typically involves using a conventional bicarbonate medium incubated at 38.5°C in 5% CO₂. However, this contrasts with the perspective of Barceló-Fimbres et al. (2015), who reported that oocyte maturation culture media supplemented with a buffer do not necessitate CO₂ gas during the maturation process and can yield superior embryo development compared to the conventional maturation system. Matthew (2011) also suggested that the medium's composition, the buffer utilized, and temperature would affect the medium's pH. These two factors interplay with the medium's pH; as the temperature increases, the pH value decreases. In light of these statements, it can be asserted that oocyte maturation culture media supplemented with a buffer do not require CO₂ gas during the maturation process, with consideration given to the temperature and the composition of the medium or buffer employed.

CELL DEVELOPMENT RATES IN CULTURE

The rates of cell (GV (*Germinal Vesicle*), GVBD (*Germinal Vesicle Breakdown*), M-I (Maturation-I) and M-II (Maturation-II)) development in various culture media were displayed in Figure 2.

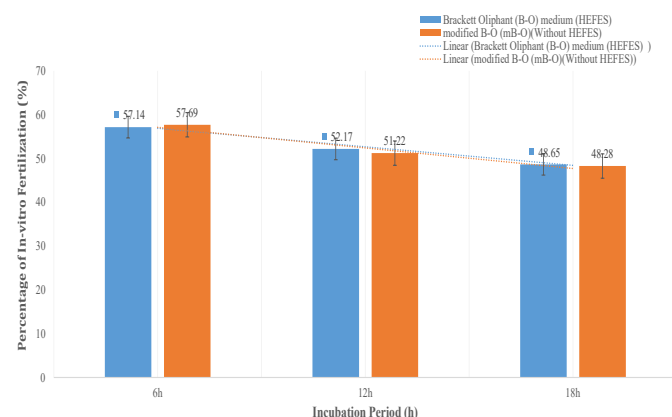


Figure 2: In-vitro fertilization rate in different culture media and various incubation periods.

Figure 2. shows that oocyte maturation results using different culture media demonstrated higher GV and M-II

development when using the mB-O medium than the B-O medium. In contrast, the B-O medium showed higher M-I development than the mB-O medium. However, the statistical analysis results indicate that both culture media used do not significantly influence cell/ oocyte development ($P>0.05$). Furthermore, the study findings reveal the absence of the GVBD stage in cell development. This phenomenon is attributed to lipolytic changes in the oocyte that resemble GVBD events, which progress to the next stage. Edwards et al. (2005) noted that a faster development rate than other phases characterizes the GVBD growth phase. Oocytes can undergo accelerated meiotic maturation and reach the M-II stage within 24h. On the other hand, Janowski et al. (2012) proposed that the follicular cells surrounding the oocyte exhibit greater competency, resulting in a higher rate of apoptosis.

The research results indicate that different culture media can enhance oocyte growth from the GV, GVBD, M-I, and M-II stages. Mature oocytes tend to have densely packed cumulus cells surrounding the zona pellucida. In line with the findings of Hassan and Kazim (2004), cumulus cells can prevent oocyte segregation, play a crucial role in supporting oocytes during the meiosis process, influence cytoplasmic maturation, and have the potential to significantly increase oocyte maturation rates (M-II).

The findings of this study illustrate that supplementing with HEPES can effectively substitute for the role of 5% CO₂ in maintaining the pH of the culture medium, thus creating optimal conditions for cell/oocyte development. Furthermore, using HEPES without 5% CO₂ facilitates oocyte development up to maturation. Oocytes that have reached the M-II stage are characterized by the formation of polar body-I, which contains homologous chromosomes (Gordon, 2003). Additionally, as indicated by Arroyo et al. (2020) and Gustina et al. (2019), the presence of polar body-1 classifies the oocyte as having reached the M-II stage, indicating oocyte maturity.

IN VITRO FERTILIZATION RATE

The average *in vitro* fertilization rates under various culture media and incubation periods are depicted in Figure 3. The results shows the average percentage of in vitro fertilization under different culture media during incubation for 6, 12, and 18 hours, which is 63.63%, 56.31%, and 62.12%, respectively. The highest fertilization percentage was achieved during the 6h incubation period, followed by a decrease of 7.32% during the 12h incubation. Additionally, during the 18h incubation, there was an increase of 5.81%. Despite the variations in these three periods, statistical analysis indicated no significant difference in the in vitro fertilization rates ($P>0.05$). The study's results reveal that the in vitro fertilization rates in different culture peri-

ods yielded nearly identical outcomes. This is attributed to the quality of oocytes and the expansion of cumulus cells. According to Moghadam et al. (2022) and Wang et al. (2009), high-quality oocytes play a substantial role in the fertilization and embryo development process. High-quality oocytes can enhance in vitro fertilization rates and result in superior embryo quality. This is corroborated by Ozturk (2020), who asserts that the quality of the oocytes employed strongly influences the success of fertilization and embryo development.

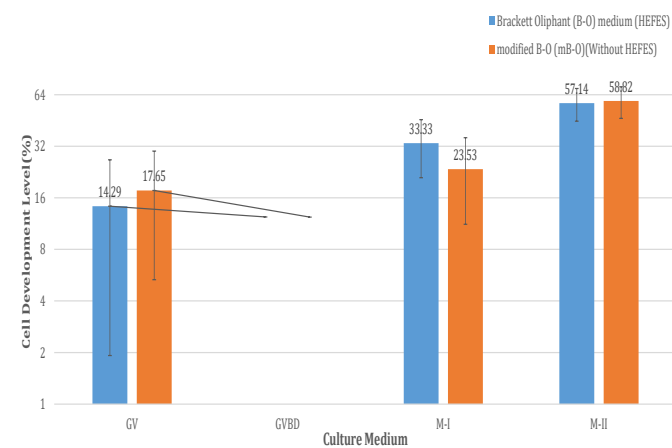


Figure 3: Cell Development Rates (GV, GVBD, M-I, and M-II) in Different Culture Media Incubated for 24 hours.

The research findings indicate that in vitro fertilization occurred during the 6h incubation period, with a rate of 63.63%. These results are consistent with those of Dode et al. (2002), who reported a sperm implantation rate of 63.3% in oocytes incubated for 6h. This is attributed to the 6h incubation period, which allows sperm to enter the oocyte that is receptive to fertilization. During this time, sperm can penetrate the cumulus cells surrounding the oocyte and fuse with its membrane, resulting in fertilization. Various factors, including the quality of both the sperm and the oocyte, influence the fertilization period. Furthermore, Gordon (2003) suggests that the timing of in vitro fertilization can impact oocyte development, as excessively long incubation periods may reduce the developmental potential of oocytes. The incubation duration is critical since the egg is receptive to sperm for only a brief period. Ovulated eggs can still be fertilized for 10-20 hours. Jaswandi (2002) reported a decrease in the in vitro sheep fertilization rate during a 12 hour incubation period. The decline during this period is attributed to eggs becoming less susceptible to fertilization within that timeframe, failing fertilization. Additionally, using different culture media during an 18h incubation period does not harm the in vitro fertilization rate and may even enhance it. Consistent with this, Nandi et al. (2002), Park et al. (2005), and Ward et al. (2002) reported that polar body-1 extrusion in cattle typically begins 16-18h after in vitro maturation. Barraud-Lange et

al. (2008) argued that a shorter gamete incubation during in vitro fertilization does not improve embryo quality compared to the standard 18-hour incubation period. The highest rates of oocyte development usually occur 5-10h after polar body-1 extrusion (Koyama et al., 2014).

This research indicates that the incubation period significantly affects the in vitro fertilization rate. The ideal incubation period for coastal cattle fertilization is 18 hour, with a success rate of 62.12%. This information is undoubtedly valuable for cattle breeders looking to optimize their in vitro fertilization success rates.

NUCLEUS DEVELOPMENT RATE

The nucleus development rate at various incubation periods in the culture medium is shown in Table 1.

The research results in Table 1. demonstrate that the statistical analysis reveals no difference in the 1PN development rate among the culture media used ($P>0.05$). However, the incubation period significantly affects 1PN development ($P<0.05$). The highest 1PN development rate was observed during the 12h incubation period, with an average rate of 36.84%, while the lowest rate was during the 6h incubation period, with an average development rate of 21.72%. A 1PN pronucleus was a zygote with only one pronucleus. The incubation period can influence pronucleus development. According to Devreker et al. (2000), Hinrichs et al. (2002), and Duijn et al. (2022), the in vitro fertilization incubation period can impact cumulus cell expansion and oocyte development after fertilization.

The findings of this study reveal that the 2PN development rate during various incubation periods was as follows: 6h (57.42%); 12h (51.70%), 18h (48.47%). The 2PN development rate decreases as the incubation period extends, with the highest rate observed at 6h, and the lowest at 18h. Statistical analysis indicates an extended incubation period significantly affects 2PN development ($P<0.05$). This can be attributed to the treatment medium's ability to provide optimal conditions for 2PN development. Several factors influence 2PN pronucleus development during incubation, including fertilization timing, incubation system, temperature, air gas composition, and ion concentrations. Consistent with the findings of Aini et al. (2016), the fertilization incubation period can impact fertilization rates and embryo development, including 2PN pronucleus development. Additionally, Fukunaga et al. (2020) state that the incubation period can influence 2PN pronucleus development. Cultural conditions can impact 2PN pronucleus development and subsequent embryo development. This is corroborated by Kobayashi et al. (2021), who consider oocytes with two pronuclei (2PN) 17-20h after fertilization as indicative of expected fertilization outcomes

Table 1: Nucleus development rate (1PN, 2PN, and >2PN) at various incubation periods in the culture medium.

No.	Culture Medium	Incubation Period (Hours)	Pronuclear Developmental (%)		
			1PN	2PN	>2PN
1.	Brackett Oliphant (B-O) medium (with HEPES, without using 5% CO ₂)	6	17.14	57.14	8.57
		12	43.48	52.17	4.35
		18	24.32	48.65	13.51
2.	Modified B-O (mB-O) medium (without HEPES, using 5% CO ₂)	6	26.92	57.69	3.85
		12	31.71	51.22	4.88
		18	24.14	48.28	13.80

, allowing embryos derived from these 2PN to undergo embryo transfer.

The research results indicate that different culture media treatments do not affect the development rate of >2PN pronuclei ($P>0.05$). However, the incubation period can influence the development of >2PN pronuclei ($P<0.05$). The highest >2PN development rate was observed during an 18h incubation, while the lowest rate is during a 12h incubation. Consistent with the findings of Nandi et al. (2002), Park et al. (2005), and Ward et al. (2002), polar body-1 extrusion in cattle typically begins 16-18h after in vitro oocyte maturation. Prolonging the incubation period can increase oocytes with more than two pronuclei (>2PN). According to Ebner et al. (2002), there is a correlation between polar body-1 morphology, fertilization, blastocyst quality, and cattle implantation and pregnancy rates.

The results of this study suggest that the utilization of HEPES in the in vitro fertilization medium is highly effective in substituting the role of 5% CO₂ for maintaining the pH stability of the culture medium to attain in vitro fertilization rates. Undoubtedly, this information was valuable for researchers aiming to improve their in vitro fertilization techniques.

CONCLUSION

The results of in vitro oocyte maturation in Pesisir cattle (M-II) using B-O and mB-O media were 57.14% and 58.82%, respectively. In contrast, in vitro fertilization using B-O and mB-O media over culture durations of 6, 12, and 18h yielded percentages of 57.14%; 52.17%; 48.65% versus 57.69%; 51.22%; 48.28%, respectively. Different culture media can significantly enhance oocyte maturation and in vitro fertilization rates in Pesisir cattle.

Based on the research results, it was clear that supplementing HEPES in the B-O medium can replace the function of 5% CO₂ and enhance oocyte maturation and in vitro fertilization rates in Pesisir cattle. Using HEPES in the B-O culture medium can provide an alternative for cell

culture outside the laboratory, making it more accessible, efficient, and cost-effective.

ACKNOWLEDGEMENTS

We sincerely thank the Research and Community Service Institute of Andalas University. It was funded by basic research in 2020 and played a crucial role in facilitating this research. Additionally, we extend our heartfelt thanks to the Laboratory of Animal Biotechnology at the Faculty of Animal Science, Universitas Andalas, for their invaluable assistance and resources throughout this study.

CONFLICT OF INTEREST

This article is free from any conflicts of interest.

NOVELTY STATEMENT

Brackett Oliphant (B-O) and modified B-O (mB-O) medium can augment oocyte maturation and in vitro fertilization rates in Pesisir cattle. The incorporation of HEPES into the B-O culture medium can serve as a substitute for 5% CO₂. This presents the prospect of conducting in vitro embryo production beyond the confines of a laboratory, eliminating the need for CO₂ dependency. Using HEPES in the B-O culture medium can be an alternative for cell culture outside the laboratory, rendering it a more accessible, efficient, and cost-effective choice.

AUTHORS CONTRIBUTION

All authors conducted the research, analyzed the data, and interpreted the research findings. Nevertheless, the manuscript was authored by Ferry Lismanto Syaiful.

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