Oocyte Division in IVF (*In Vitro Fertilization*) with Cauda Epididymis Sperm of Bali Cattles

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Abstract. *This study aimed to investigate the fertilization rate of oocytes using sperm from the cauda epididymis of Bali cattle until cleavage. The research spanned 10 months, involving various stages conducted at different facilities. Testicular samples were obtained from the Cibinong Slaughterhouse, followed by freezing of epididymal sperm at the Reproductive Rehabilitation Unit laboratory of the Faculty of Veterinary Medicine, Bogor Agricultural Institute. Post-thaw analysis of frozen sperm was performed at the Reproduction, Breeding, and Animal Cell Culture facilities of the LIPI Cibinong Biotechnology Research Center. At the same time, the in vitro fertilization (IVF) process took place at the Cipelang Bogor Livestock Embryo Center. The primary objective of this investigation was to assess the fertility of cauda epididymal sperm post-thawing, specifically evaluating the impact of caffeine supplementation on the success of in vitro fertilization (IVF). The methodology involved collecting cauda epididymal sperm at the slaughterhouse, with subsequent analysis of sperm quality after thawing by adding different concentrations of caffeine (T0: 0 mg/ml, T2: 2 mg/ml, T4: 4 mg/ml, T6: 6 mg/ml) in four replicates. The key parameter examined was the fertility of cauda epididymal sperm using the IVF technique. The experimental design utilized a one-way Completely Randomized Design (CRD), followed by Tukey-W-Procedure testing using SPSS 16. The findings revealed that the fertility of cauda epididymal sperm at concentrations T2 and T4 was significantly higher ($P<0.05$) compared to T0 and T6. Consequently, the study concluded that the addition of 4 mg/ml caffeine to thawed cauda epididymal sperm resulted in a 37.50% success rate in the IVF process and a 10% cleavage rate of oocytes in Bali cattle.*

Keywords*: cauda epididymis, oocyte division, fertility, IVF*

1. INTRODUCTION

An unfortunate incident occurred when a male Bali cattle, possessing exceptional semen quality, succumbed suddenly to a JD attack. Despite this, the animal maintained good body condition. Given the circumstances, it becomes imperative to provide special attention to such livestock, with a focus on preserving or utilizing their reproductive organs, particularly the testicles. The testes serve as the primary site for sperm production, crucial for fertilization processes. Even in deceased cattle, sperm within the testicles remains viable for further processing. Notably, sperm within the epididymis retains good quality postmortem (PM) as long as there is a consistent supply of adenosine triphosphate (ATP) as an energy source, typically within 8-12 hours PM in cattle, as noted by Sutardi (1987, cited in Gumilar, 2011). The cauda epididymis houses mature sperm, making it a pivotal source for sperm isolation. Following isolation, sperm undergoes a freezing process, facilitating its use in Artificial Insemination (AI). This approach enables the preservation of the genetic traits of superior males, with the aspiration of maintaining their fertility and quality in fertilizing egg cells, both in vivo and in vitro.

Reduced sperm quality may manifest during the freezing and storage processes. Enhancing sperm quality post-thawing necessitates using stimulants to activate the motility of surviving sperm cells during freezing, consequently augmenting fertility. Caffeine emerges as a promising additive for this purpose. As an alkaloid, caffeine exerts its effects as a stimulant on the

central nervous system (CNS) and metabolism in humans. Recent studies have explored its application in animal sperm solutions to enhance both sperm motility and fertility. The mechanism behind this enhancement lies in caffeine's ability to inhibit adenosine receptors, suppress phosphodiesterase activity, and modulate intracellular calcium exchange (Lamarine, 1998). These actions lead to alterations in the intracellular concentration of cyclic adenosine monophosphate (cAMP), thereby promoting sperm motility (Gerhastuti, 2009).

Enhancing sperm quality is important to improve fertility outcomes. However, the freezing and thawing process often leads to reduced sperm motility and viability. This study specifically targets the post-thawing period, aiming to preserve the quality of sperm extracted from the epididymis after freezing by administering caffeine in the medium.

The findings from research utilizing sperm isolation from the epididymis are anticipated to offer a solution for male livestock with superior semen quality that succumb to infectious diseases. Furthermore, it is envisaged that these research outcomes will contribute to the development of animal husbandry practices overall, particularly in the preservation of genetic resources through the application of in vitro fertilization (IVF) technology, thereby benefiting rare livestock breeds specifically.

2. RESEARCH METHOD

Sperm colection

Testes were obtained from 12 male Bali cattle and subsequently cleaned from the surrounding skin. The testicles were then sectioned into four parts - the corpus, head, cauda, and vas deferens - using arterial forceps. Following this, they were immersed in a physiological solution of 0.9% NaCl supplemented with streptomycin (0.1 g/l) and penicillin (0.06 g/l). The prepared samples were placed in a cool box containing warm water at 37°C for transportation to the laboratory. The cauda epididymis was separated from the testis in the laboratory,, and its tissue was incised. The white fluid from the cauda epididymis was then aspirated using a micropipette, transferred onto a glass slide, and subjected to analysis to determine its characteristics.

Sperm with good characteristics will undergo additional processing to be turned into frozen semen, which will then be stored in liquid nitrogen at a temperature of -196°C. Subsequently, the next step involves conducting fertility tests for each caffeine treatment, including T0 (0 mg/ml), T2 (2 mg/ml), T4 (4 mg/ml), and T6 (6 mg/ml).

In vitro fertilizatiom

Ovarian collections from slaughterhouses were stored in Ringer's lactate (LR) medium plus the antibiotic streptomosin (1000 µl in 100 ml) at room temperature (25oC). The ovaries are washed and cleaned with LR then put back in LR, placed on a hot plate at a temperature of 38oC. Aspirate oocytes from the ovaries using an 18 G syringe and needle filled with PBS, the aspiration results obtained are collected in a 100 x 100 mm petri dish (Saito, 2005).

Oocytes were washed (from a 100 x 100 mm petridish) and evaluated for their quality. Quality A (oocytes covered with cumulus cells), B (oocytes covered with thin cumulus), C (not covered with cumulus cells), and D (cumulus cells are damaged or degenerated). The collected oocytes were collected in 10 x 35 mm petri dishes in PBS media. In the research, only quality A and B oocytes were used, then placed in maturation media which had been covered with mineral oil, then rinsed to remove the remaining PBS media, after rinsing twice, placed in a drop of maturation media (TCM-199) which was covered with mineral oil, then stored in an incubator with a temperature of 38.5oC and 5% CO2 for 18-22 hours (Saito, 2005).

Prepare fertilization media, namely Brackett Oliphant (BO) solution. Preparing sperm: frozen cauda epididymis sperm (from stage II) is thawed at a temperature of 38oC and then centrifuged at a speed of 1800 rpm for 5 minutes, then washed 2 times. Dilute sperm with Tris (2.1 ml) plus caffeine with treatment T0 (0 mg/ml), T2 (2 mg/ml), T4 (4 mg/ml), and T6 (6 mg/ml), then make a drop The sperm for each drop contains approximately 2 million sperm and is covered with mineral oil. Oocytes that have been matured are washed with oocyte washing media, namely OWS. Fertilization: Oocytes that have been matured are inserted into sperm drops that have been treated with caffeine, where 10-20 oocytes are inserted into each drop, then placed in an incubator equipped with a temperature of 38.5oC and 5% CO2, the fertilization process takes between 5-18 hours

Analisis in vitro fertilisasi (IVF)

IVF success rate analysis was carried out using 1% aceto-orcein staining. Oocytes that had been cultured for 18 hours were washed in PBS medium until the cumulus cells disappeared using a Pasteur pipette, then placed in an object glass and covered with a cover glass, without touching the oocyte drop before pressing on the sides. Pressure while viewing using a microscope so that the oocyte does not break. The space between the cover glass is filled with fixation solution, absorb the excess with a tissue carefully so that the oocyte does not come out. The object glass containing the oocytes was soaked in the fixation solution for 48 hours.

The object glass was removed from the fixation solution after 48 hours. The remaining solution was removed by applying methanol from the edge and left to dry. The object glass is placed horizontally then stained with 1% aceto-orcein through one edge of the cover glass. Wait about 5-10 minutes for the oocytes to be completely colored. Excess dye can be reduced with aceto glycerin solution, by flowing it over the edge of

the cover glass, and the remaining solution is cleaned with a tissue and protected using neutral colored nail polish, waited until it dries then observed using a microscope.

3. RESULTS AND DISCUSSION

Fertilization involves the fusion of sperm cells and egg cells. Following fertilization, the initial developmental stages include the formation of a zygote, followed by mitotic division resulting in the formation of two cells, which then progress into a morula and subsequently a blastocyst. The fertility of cauda epididymal sperm was assessed by examining zygote development at 18 hours and cell division at 36 hours post in vitro fertilization, utilizing 1% aceto-orcein staining. The fertility of cauda epididymal sperm from Bali cattle post-thawing is presented in Table 1.

Table 1. Percentage of cauda epididymal sperm fertility after thawing with caffeine treatment at 18 hours after in vitro fertilization

Caffeine (mg/ml)	Number of oocytes	Fertilized (%)	Not fertilized (%)
T0	68	19.12 (13/68)[b]	80.88 (55/68)
T2	72	27.78 (20/72)[a]	72.22 (52/72)
T4	80	37.50 (30/80)[a]	62.50 (50/80)
T6	80	13.75 (11/80)[b]	86.25 (69/80)

Note: different superscripts in the same column indicate significant differences P<0.05). T0=0mg/ml, T2=2mg/ml, T4=4mg/ml dan T6=6mg/ml.

The Advanced test results with Tukey test indicated that the fertility percentage of cauda epididymal sperm from Bali cattle after thawing was significantly higher (P<0.05) at T4 compared to T0 and T6. Although, there was no significant difference (P>0.05) compared to T2. This suggests that caffeine can enhance Ca2+ fluctuations within oocytes, which commence upon sperm fusion. These Ca2+ fluctuations within the endoplasmic reticulum are regulated by inositol triphosphate (IP3). The elevation in Ca2+ flow persists during fertilization, extending until the formation of the pronucleus and the onset of mitotic division in the zygote, as observed in studies by Jones (2007), Gordon (2003), and Oehninger and Franken (2006).

Table 2 Cell division at 36 hours after in vitro fertilization

Caffeine (mg/ml)	Number of fertilized oocytes	Cell division (%)	
		2 cells	4 cells
T0	13	100 (13/13)	0 (0/23)
T2	20	95 (19/20)	5 (1/20)
T4	30	90 (27/30)	10 (3/30)
T6	11	100 (11/11)	0 (0/11)

Note: T0=0mg/ml, T2=2mg/ml, T4=4mg/ml dan T6=6mg/ml.

The inclusion of caffeine in T6 (6 mg/ml) exhibits a detrimental impact, as evidenced by the reduced fertility of cauda epididymal sperm after thawing, as shown in Table 2. This adverse effect arises from the introduction of caffeine in T6 (6 mg/ml), which induces uncontrolled hyperactivation of motility, thereby triggering rapid capacitation. Consequently, this rapid capacitation leads to the detachment of the plasma and outer membrane of the acrosome, as discussed by Garner and Hafez (2000). The detachment of the acrosomal membrane renders sperm incapable of fusing with the zona pellucida due to the absence of the hyaluronidase enzyme. The absence of acrosomal enzymes renders the sperm non-functional, as explained by Elder and Dale (2003), as the energy reserves are depleted. Consequently, this leads to the immotility of sperm due to the cessation of fibrous contractions, which are responsible for regulating cAMP phosphorylation, as elucidated by Soeparna and Nurcholidah (2014) and Conner and Barratt (2006).

Following fertilization, the zygote undergoes mitotic division, progressing from 2 cells to eventually form a blastocyst. Table 2 displays the percentage of 2-cell divisions, with T0 showing 100%, followed by T6 (100%), T2 (95%), and T4 (90%). This indicates that both the control and caffeine-treated groups can undergo fertilization up to 2-cell divisions on the first day. However, only in T4 (4 mg/ml) and T2 (2 mg/ml) can fertilization progress to 4-cell divisions by the second day. This suggests that caffeine at concentrations of 2 mg/ml and 4 mg/ml can enhance Ca2+ fluctuations in the oocyte, leading to the formation of pronuclei and the division of 4 cells. The absence of embryo development at T6 may be attributed to high caffeine concentrations causing uncontrolled Ca2+ fluctuations, resulting in failed cell division. Similarly, embryos failing to develop at T0 may be due to the absence of Ca2+ regulation in the oocyte, influenced by factors such as sperm concentration, medium pH, and culture conditions, including temperature, O2, and CO2 levels, as discussed by Boediono et al. (2000). The success of in vitro fertilization is significantly influenced by factors such as sperm concentration (ranging from 1-3 million sperm/ml), medium pH of 7.4, culture temperature of 38°C, and O2 concentration of 5% and CO2 concentration of 5% (Lazzari et al, 1998 in Gordon, 2003).

The efficacy of fertilization is significantly iinfluenced by the quality of both sperm and egg cells. Sperm preserved in liquid nitrogen typically undergoes a decrease in motility by 50-60% and viability by 20-30 (Sukmawati et al, 2014). However, the addition of caffeine to cauda epididymal sperm after thawing has the potential to enhance fertility. This is attributed to caffeine's ability to induce the release of intracellular calcium and inhibit phosphodiesterase (PDE) activity, consequently elevating cyclic adenosine monophosphate (cAMP) levels in sperm (Hasbi et al, 2011; Lamarine, 1998)

4. CONCLUSION

The research findings conclude that the success rate of the in vitro fertilization process and the division of oocytes into 4 cells using cauda epididymal sperm after thawing, with the addition of 4 mg/ml caffeine, stands at 37.50% and 10%, respectively.

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