Research Article

# Quality of Aceh Cattle Ovaries Added with Bovine Pituitary Extract in the Vitrification Medium

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**Abstract** | Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) hormones are often added to ovarian vitrification media to prevent apoptosis of granulosa cell follicles. Bovine pituitary has been proven as an effective substitute for the functions of FSH and LH in some studies related to superovulation due to its high content of both hormones. Therefore, this study aimed to determine the effect of bovine pituitary extract (BPE) supplementaion to vitrification media. A total of 18 ovaries were usedwhich was divided into two groups: the vitrification medium group without Bovine Pituitary Extract (BPE, Gibsco™) (1 ml PBS + 0.5 M sucrose + 30% ethylene glycol) and with BPE addition (1 ml PBS + 0.5 M sucrose + 30% ethylene glycol + BPE 30 µg/mL). Each group was separated into three vitrification times of 0, 7, and 14 days with three replications. The results showed that adding BPE to the vitrification medium increased the number of intact follicles (P<0.05) at each development stage, with the highest number occurring in the primordial and primary follicle stages. The number of primordial and primary follicles on day 14 of vitrification the control group vs the BPE group was 62.08±18.25 vs 72.72±5.67 and 45.62±15,9 vs 65.78±7.17, respectively. In conclusion, the addition of BPE to the vitrification medium could increase the number of intact follicles, preventing damage that may occur during the vitrification process.

**Keywords** | Aceh cattle, Bovine pituitary extract, Follicle, Reproductive technology, Vitrification

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## INTRODUCTION

Aceh cattle are one of the local livestock breeds in Indonesia playing a crucial economic role for a significant population in the Aceh Province. However, there is a risk of decline due to the uncoordinated slaughter of female cattle. To address this issue, assisted reproductive technology offers a promising solution, allowing for maximized utilization of ovaries from economically valuable or endangered animals in *in-vitro* embryo production (IVEP) programs.

IVEP has successfully addressed productivity and infertility issues in both animals and humans. Several studies showed that the success of IVEP depends on oocyte quality (Romaguera *et al*., 2010; Catalá *et al*., 2012). Ovaries obtained from the slaughterhouse can only last for two to four hours (Febretrisiana *et al*., 2015). Proper storage before processing allows for the production of well-developed oocytes *in vitro*. Meanwhile, vitrification is a freeze-storage method applicable to ovaries without distance and time constraints. It is a cryopreservation method without ice crystal formation, rapidly passing through perilous temperature zones with a very short exposure time, reducing cell structure damage (Cuello *et al*., 2004). Vitrified ovarian tissue tends to have better oocyte morphology compared to conventional cryopreservation methods (slow freezing) (Campos *et al*., 2016).

Several factors can affect the quality of ovarian tissue undergoing vitrification, such as metabolic damage due to dehydration, osmotic shock, and the thawing process. According to Fathi *et al*. (2013), a supportive environment is needed to protect follicles during vitrification, resulting in a decrease in the number of deaths due to cryo injury. Zhang *et al*. (2013) and Jain and Paulson (2016) mentioned that follicle-stimulating hormone (FSH) plays a crucial role in the growth and development of follicles, specifically as an anti-apoptotic factor in granulosa cell follicles. Thomas and Vanderhyden (2006) found that granulosa cells are essential for follicle growth and development, while atresia is triggered by cells undergoing apoptosis. Moreover, the addition of FSH to vitrification media by Yang *et al*. (2015) proved to maintain follicle quality.

Another gonadotropin secretion also contributing to follicle maintenance is luteinizing hormone (LH) which has been proven to preserve the function of vitrified ovaries (Zheng *et al*., 2020). FSH and LH are gonadotropin hormones produced by the anterior pituitary gland. The use of pituitary extract of Aceh cattle as a source of gonadotropin hormones in estrus synchronization and superovulation has been carried out (Hafizuddin *et al*., 2010; Siregar *et al*., 2013, 2020). However, the use of pituitary extract in vitrification media has not been reported. Therefore, this study aimed to examine the quality of Aceh cattle ovaries vitrified in media added with pituitary extract using different concentrations and storage durations.

## MATERIALS AND METHODS

### Experimental design

In this study, 18 ovaries were obtained from the slaughterhouse in Banda Aceh. The treatments were divided into two groups: the vitrification medium group without the addition of Bovine Pituitary Extract (BPE, Gibsco™) and with the addition of BPE (Talbot and Powell, 2004). The composition of each vitrification medium was 1 ml PBS + 0.5 M sucrose + 30% cryoprotectant EG (the vitrification medium group without the addition of BPE) and 1 mL PBS + 0.5 M sucrose + 30% cryoprotectant EG + BPE 30 µg/mL (the vitrification medium group with the addition of BPE). Each group was further separated into three vitrification times of 0, 7, and 14 days with three replicates.

### Procedure

#### Ovary preparation

Ovaries obtained from the slaughterhouse were collected after the cattle were slaughtered and promptly transported to the Reproduction Laboratory, Faculty of Veterinary Medicine, Syiah Kuala University. The collected ovaries were cleaned with PBS, then placed in an ovarian collection container containing 0.9% physiological NaCl supplemented with streptomycin and penicillin. Next, the ovaries in the collection container are placed in a collection flask at a temperature of 30-35 °C. The preparation of ovaries after collection and ovarian handling upon arrival at the laboratory was conducted following the procedure outlined by Campos *et al*. (2016).

#### Ovary vitrification

Ovary vitrification was performed based on the oocyte vitrification method by Djuwita *et al*. (2005) with slight modifications. The ovaries were sequentially exposed to solutions of PBS + 0.25 M sucrose and PBS + 0.5 M sucrose, each for 5 minutes. The samples were then placed into the vitrification medium consisting of PBS (Supelco, Germany), sucrose (Sigma-Aldrich, USA), and Ethylene glycol (Sigma-Aldrich, USA). The ovaries along with the medium were packaged in 50 m tubes, exposed to liquid nitrogen for 10 seconds, and immersed in liquid nitrogen for 0, 7, and 14 days. Samples were taken from liquid nitrogen, left in the air for 10 seconds, and immersed in water at 37°C until thawed. The ovaries were sequentially placed in solutions of PBS + 0.5 M sucrose, PBS + 0.25 M sucrose, and PBS three times fo 5 minutes each time.

#### Histological examination

Post-vitrification ovaries were subjected to histological examination by initially dehydrating in alcohol, clearing in xylene, and embedding in paraffin. Tissues were serially sectioned using a rotary microtome with a thickness of 5 µm, then the sections were fixed to glass slides and stored in an incubator at 37°C for 24 hours. Subsequently, the preparations were stained with hematoxylin-eosin (HE).

An evaluation was performed by counting the number of primordial, primary, secondary, and antral (tertiary and de Graaf) follicles which show normal morphology in each

field of view. Primordial follicles consisting of an oocyte surrounded by a single layer of flattened cells and primary follicles composed of an oocyte surrounded by a single layer of cuboidal cells (unilaminar). Secondary follicle slightly similar to primary follicle but consisting more than one cuboidal cell layer (multilaminar). The final stage of this follicle marks the formation of a small antrum. Tertiary follicles are characterized by an enlarged antrum, while de Graaf follicles are marked by a separated oocyte and a very large antrum (Myers *et al*., 2004). The counting of each follicle was performed only once when the section intersected the nucleus. A total of six different sections were counted for each ovarian sample at intervals of 10 while secondary and tertiary follicles was calculated based on the presence of the oocyte nuclear offspring to avoid double counting.

### Statistical analysis

The data obtained in percentage form were analyzed using analysis of variance (ANOVA) and subsequently subjected to the Duncan Multiple Range Test using SPSS 25 for Windows.

## RESULTS AND DISCUSSION

Ovarium yang digunakan dalam penelitian ini adalah ovarium sapi Aceh dengan bobot dan morfometri yang relative sama (Table 1). The number of surviving follicles was counted using HE staining to determine the protective effect of BPE on ovarian follicles after vitrification, as presented in Figure 1. Primordial follicles contained small oocytes with a single layer of flattened granulosa cells but no zona pellucida. Meanwhile, primary follicles contained pale small oocytes with a characteristic zona pellucida and a single layer of cuboidal granulosa cells. Secondary follicles were surrounded by irregular spaces resulting from the differentiation of ovarian stromal cells (Songsasen *et al*., 2009). These epithelial cells then formed the follicle theca and in the final development of secondary follicles, the separation of the follicle theca into internal and external theca occurred. After the separation, cavities (antrum) appeared within the layer of granulosa cells. In line with the increasing number of antrums corresponding to follicle growth, the boundary gradually disappeared, leaving only one large antrum forming a tertiary follicle (Syafruddin *et al*., 2023).

**Table 1:** Average weight and morphometric size of ovaries of Aceh cattle.

| Ovary | Ovarian weight (g) | Ovary length (mm) | Ovary width (mm) |
|---|---|---|---|
| Right | 4.83±2.61 | 23.51±4.00 | 16.67±3.19 |
| Left | 4.67±2.46 | 23.05±4.63 | 15.89±4.36 |

Not significant differences (P>0.05).

In this study, the most common stage found in each treatment group was the primordial follicle, as presented in Table 2. This result was consistent with the report by Campos *et al*. (2016), stating that the largest proportion in the ovaries of cattle undergoing vitrification or slow freezing was primordial follicles. Furthermore, Kim *et al*. (2009) stated that primordial and primary follicles were responsible for initiating hormone production.

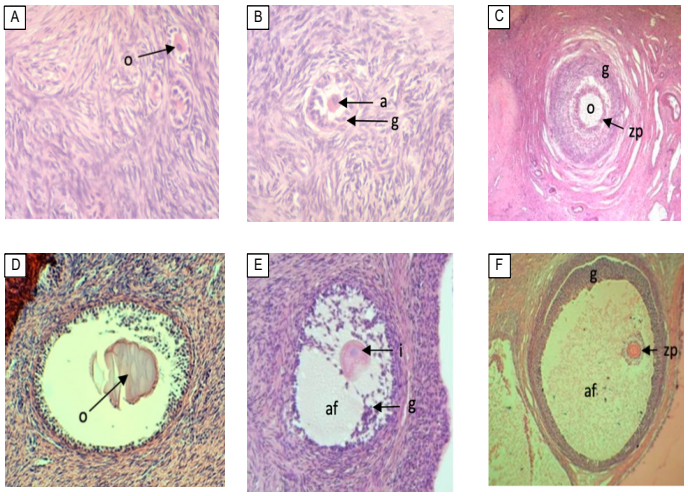


**Figure 1:** Various stages of follicle development in Aceh cattle ovaries after vitrification with BPE: A: primordial follicle; B: primary follicle; C: secondary follicle with oocyte; D: damaged secondary follicle with oocyte; E: tertiary follicle; F: de Graaf follicle (o: oocyte; g: granulosa cell; zp: pellucida zone; af: antrum folliculi). Scale bar=200 µm. Magnification 10x.

**Table 2:** The average number of follicles at each developmental stage in ovaries vitrified with the addition of 30 µg/mL BPE with different vitrification durations.

| Stadium | 0 days | | 7 days | | 14 days | |
|---|---|---|---|---|---|---|
| | Control (n=3) | BPE (n=3) | Control (n=3) | BPE (n=3) | Control (n=3) | BPE (n=3) |
| Primordial follicles | 62.74±0.89$^{a}$ | 75.75±1.29$^{a}$ | 62.14±3,28$^{a}$ | 69.84±5.98$^{a}$ | 62.08±18.25$^{a}$ | 72.72±5.67$^{b}$ |
| Primary follicles | 57.0±0.87$^{a}$ | 60.36±0.10$^{a}$ | 61.81±9.45$^{a}$ | 71.68±8.27$^{a}$ | 45.62±15.9$^{a}$ | 65.78±7.17$^{b}$ |
| Secondary follicles | 22.7±6.39$^{a}$ | 62.5±3.46$^{b}$ | 38.98±9,46$^{a}$ | 64.78±12.16$^{b}$ | 32.58±14.17$^{a}$ | 51.78±22.33$^{b}$ |
| Antral follicles | 42.85±1.64$^{a}$ | 60.0±5.77$^{b}$ | 21.42±5.77$^{a}$ | 50.0±9.62$^{b}$ | 33.33±19.24$^{a}$ | 66.67±9.62$^{b}$ |

a, b, c Different superscripts in the same rows indicate significant differences (P<0.05).

In general, the addition of BPE in the vitrification medium increased the number of intact follicles at each development stage. The ability of BPE correlated with the addition of FSH in the vitrification medium. This result supported the finding reported by Yang *et al*. (2015), Ma *et al*. (2017), and Wang *et al*. (2023), that the addition of FSH in the medium increased blood supply to the transplanted ovaries, enhanced the survival rate of follicles, and inhibited apoptosis of ovarian cells. The ability to increase the survival rate of follicles was attributed to the inhibition of excessive autophagy caused by oxidative stress, similar to the potential showed by FSH (Shen *et al*., 2017). This result proved that BPE has similar capabilities to FSH in several reproductive aspects. Recent studies showed that pituitary extract can enhance livestock productivity (Amiruddin *et al*., 2014; Outang *et al*., 2017; Sayuti *et al*., 2022).

## CONCLUSIONS AND RECOMMENDATIONS

The addition of BPE to the vitrification medium could increase the number of intact follicles, preventing damage that may occur during the vitrification process.

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## NOVELTY STATEMENT

This research is the first to determine the effect of giving BPE for vitrification the ovaries of Aceh cattle.

## AUTHOR'S CONTRIBUTION

TNS and CIN: Conceptualization; CIN, NWKK, TNS, and KE: Methodology, formal analysis, and investigation; CIN: Data processing. TNS, CIN, AS: Writing original draft preparation, writing review and editing. All authors have read and agreed to the published version of the manuscript.

### Conflict of interest

The authors have declared no conflicts of interest.

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