

## Research Article



# Potential of Bovine Pituitary Extract for Superovulation Based on Increased Expression of Estrogen Receptor Alpha and Progesterone Receptor in Local Rabbit Uterine Tissue

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**Abstract** | The bovine pituitary contains follicle-stimulating hormone (FSH) and luteinizing hormone (LH), both of which can be used in superovulation. Successful superovulation can be evidenced by the presence of estrogen and progesterone receptors in the uterus. The aim of this study was to evaluate the potential of bovine pituitary extract (BPE) in inducing superovulation, based on the distribution and expression of estrogen receptor alpha (ER $\alpha$ ) and progesterone receptor-A (PR-A) in rabbit uterine tissue. Six local female rabbits, weighing between 1.8–2.2 kg and having previously given birth, were used in this study. The rabbits were divided into two treatment groups (n=3): KL1, the control group, which was injected with physiological NaCl (2.6 ml total), and KL2, the treatment group, which was injected with BPE (2.6 ml total). Both groups received injections of physiological NaCl or BPE five times at 12-hour intervals. The injection volumes for each rabbit were as follows: 1 ml for the first injection, 0.5 ml for the second and third, and 0.3 ml for the fourth and fifth. Twelve hours after the final BPE injection, KL2 rabbits were administered 100 IU hCG and mated with male rabbits. KL1 rabbits were mated without hCG administration. On the 6th day after treatment, the rabbits were euthanized, and their uterine organs were collected, processed histologically, and stained using immunohistochemical staining with the avidin-biotin complex peroxidase (ABC) method. The distribution of ER $\alpha$  and PR-A in uterine tissue was analyzed descriptively. Differences in receptor expression between the two groups were assessed based on intensity scores, using the Mann-Whitney U test. The results showed the presence of both ER $\alpha$  and PR-A in the uterine glands, lamina epithelialis, and lamina muscularis of the rabbit endometrium. A significant difference in ER $\alpha$  expression was observed between KL1 and KL2 rabbits (P<0.05), while the difference in PR-A expression was highly significant (P<0.01). In conclusion, BPE treatment significantly increased the expression of ER $\alpha$  and PR-A in the endometrial layer of the rabbit uterus, suggesting that BPE can be used as an indicator of successful superovulation.

**Keywords** | Bovine pituitary extract, ER $\alpha$ , PR-A, Local rabbit, Superovulation, Uterus

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Hormones commonly used in superovulation protocols include gonadotropins such as follicle-stimulating hormone (FSH) and pregnant mare's serum gonadotropin (PMSG). Each hormone has its own advantages and disadvantages. FSH typically produces a better ovarian response compared to PMSG, leading to more ovulated ova, fewer anovulatory follicles, and higher-quality embryos. However, FSH has some drawbacks, including its relatively high cost due to limited availability in the domestic market. Additionally, repeated administration can induce stress in animals, potentially resulting in reduced embryo quality. On the other hand, PMSG has a longer duration of action, with a half-life of approximately 2-5 days, and its residues remain in the bloodstream for up to 10 days (Putro, 1996). This prolonged half-life can lead to wide variations in superovulation responses, persistent follicles in the ovaries, hormonal imbalances, and non-viable embryos for transfer (Afriani *et al.*, 2020).

An alternative to overcome the challenges of using these two gonadotropins is the use of pituitary extract. Some studies have reported that pituitary extract can induce estrus in dairy cattle and increase reproductive efficiency in goats (Siregar *et al.*, 2013). Intramuscular injection of pituitary extract in kaligesing goats can induce early estrus behavior and enhance estrus behavior performance in e tawah goats (Setiawan *et al.*, 2019). Hafizuddin *et al.* (2010) also demonstrated that pituitary extract and PMSG have the same effectiveness in inducing superovulation in mice. Nalley *et al.* (2017) reported that pituitary extract has advantages over synthetic hormones, as it produces gonadotropins such as FSH, luteinizing hormone (LH), and growth hormone (GH), which plays a role in fetal growth and increases milk production in mothers. The gonadotropins and GH present in pituitary extract are natural and do not negatively affect the reproductive performance of donor females.

The use of pituitary extract for superovulation is still limited (Arum *et al.*, 2013; Sayuti *et al.*, 2022), so optimal methods and dosages have not yet been established. Sayuti *et al.* (2022) reported that administering bovine pituitary extract (BPE) to rabbits once daily for three days, with decreasing doses of 1; 0.5; and 0.3 ml, did not show significant differences in estrogen and progesterone concentrations or the number of fetuses produced compared to the control group, which received normal saline. In other species, the use of BPE for superovulation has successfully increased the number of corpus luteum and embryo acquisition in Aceh cattle (Arum *et al.*, 2013) and increased the number of births in local Aceh goats (Siregar *et al.*, 2013). It is believed that BPE has similar superovulation-inducing properties to FSH. Therefore, the treatment protocol should follow the pattern of FSH administration.

Several variations in FSH dose and injection frequency for rabbit superovulation have been reported. Zhang *et al.* (2017) used a dose of 30 IU/rabbit administered in six injections over three consecutive days. According to Kaufman *et al.* (1998), administering FSH for four days to induce superovulation in rabbits can improve reproductive biotechnology efficiency following embryo cryopreservation. However, Techakumphu *et al.* (2002) reported no differences in the number of ovulations and developing follicles in New Zealand White (NZW) rabbits induced with FSH at doses of 21, 28, and 40 mg, administered in five injections at 12-hour intervals. In this study, the administration of bovine pituitary extract will follow a five-injection pattern.

Superovulation success can be indicated by an increased number of offspring (Nur *et al.*, 2016), concentrations of estrogen and progesterone (Amiruddin *et al.*, 2014), and an increase in the number of corpora lutea (Siregar *et al.*, 2020). According to Meikle *et al.* (2001), hormones such as estrogen and progesterone exert physiological effects when they interact with their receptors in the tissues of target organs, such as reproductive organs. These receptors include estrogen receptors (ER) and progesterone receptors (PR). One isoform of ER, besides ER beta (ER $\beta$ ), is estrogen receptor alpha (ER $\alpha$ ), which plays a key role in the physiological functions of the female reproductive tract. ER $\alpha$  is found in the ovaries, mammary glands, uterus, testes, pituitary gland, hippocampus, kidneys, epidermis, and adrenal glands (Rai and Jeswar, 2010). Progesterone receptors also have two isoforms, PR-A and PR-B (Kastner *et al.*, 1990).

Hormone receptor expression in tissues can be detected using immunohistochemistry (IHC). The principle of IHC is to detect the binding between antigens in tissues and exogenous antibodies. The distribution of ER $\alpha$  and PR in bovine oviduct tissue during follicular and luteal phases has been detected using IHC techniques (Saruhan *et al.*, 2011). IHC techniques have also been applied to detect the expression and distribution of PR-A and PR-B in bovine oviducts during the estrous cycle and pregnancy (Saint-Dizier *et al.*, 2012). However, the distribution of ER $\alpha$  and PR-A in the uterine tissues of rabbits induced with BPE for superovulation has not been reported. Therefore, the aim of this study was to evaluate the potential of BPE in inducing superovulation, based on the distribution and expression of ER $\alpha$  and PR-A in rabbit uterine tissue.

## MATERIALS AND METHODS

In this study, six local female rabbits, which had previously given birth and weighed between 1.8-2.2 kg, and one local male rabbit were used. Prior to treatment, the rabbits were acclimated for thirty days in separate cages (Kanayama *et al.*, 1995). The rabbits were provided feed and water *ad li-*

*bitum*. The rabbits were divided into two treatment groups (n=3): KL1, as the control group, consisted of local rabbits injected with physiological NaCl with a total dose of 2.6 mL, and KL2, which consisted of local rabbits injected with bovine pituitary extract (BPE) with a total dose of 2.6 mL. The doses were given refers to previous research conducted by [Hafizuddin et al. \(2024\)](#).

### PREPARATION OF PITUITARY EXTRACT

Bovine pituitary glands were collected from a slaughterhouse in Banda Aceh. The collected pituitary glands were placed in a thermos containing ice and immediately brought to the laboratory. The pituitary glands were cleaned of connective tissue and separated from the outer membrane, then sliced to a thickness of  $5 \pm 1$  mm. The pituitary extract was prepared based on the method applied by [Isnaini et al. \(1999\)](#). The content of FSH and LH hormones in the extracted bovine hypophysis was  $40.355 \pm 20.75$  and  $10.257 \pm 5.74$  mIU/mg, respectively ([Novita et al. 2024](#)).

### SUPEROVULATION INDUCTION AND MATING

The first injection of physiological NaCl in KL1 and BPE in KL2 was administered at 20:00 WIB and repeated every 12 hours for a total of five injections. Normal saline in KL1 and BPE in KL2 were administered as follows: 1 mL (1<sup>st</sup> injection), 0.5 mL (2<sup>nd</sup> injection), 0.5 mL (3<sup>rd</sup> injection), 0.3 mL (4<sup>th</sup> injection), and 0.3 mL (5<sup>th</sup> injection). Twelve hours after the last BPE injection, the rabbits in KL2 were injected with 100 IU of hCG and mated with the male rabbit modified from ([Techakumphu et al., 2002](#); [Sayuti et al., 2022](#)), while the rabbits in KL1 were mated without hCG injection.

### UTERUS COLLECTION

On the 7<sup>th</sup> day after treatment, the rabbits in KL1 and KL2 were terminated by slaughter. The right and left uterine horns were collected, prepared, and processed using histological techniques for histological slide preparation.

### HISTOLOGICAL SLIDE PREPARATION

Histological preparation of the uterus followed the histotechnical method referred to by [Kiernan \(2015\)](#) with modifications. The fixation process was performed by immersing the entire uterus in NBF10% solution for 7 days. After fixation, the uterus was transferred into 70% alcohol as a stopping point until dehydration. After the fixation process, the uterus was cut longitudinally (mirror section) and then inserted into a tissue cassette that had been coded. Samples were immersed sequentially in alcohol with graded concentrations (70%, 80%, 90%, 95%, and absolute (3 replicates). Then the clearing process was carried out using silol solution, followed by tissue infiltration using liquid paraffin. After the infiltration process, the samples were embedded in liquid paraffin and then moulded into tissue blocks.

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### IMMUNOHISTOCHEMISTRY STAINING AND IDENTIFICATION OF STAINING RESULTS

The ABC immunohistochemistry staining procedure was carried out by following the manual protocol for the mouse and rabbit specific HRP/DAB (ABC) detection IHC kit (Abcam®). IHC staining procedure of ABC method refers to the procedure manual of mouse and rabbit specific HRP/DAB (ABC) detection IHC kit (Abcam®). Before the IHC staining was started, deparaffinisation was first carried out with silol solution (3 repetitions) and then the process of rehydrating the tissue slides by soaking the slides in absolute alcohol (3 repetitions), 95%, 90%, 80%, and 70%. Slides were washed with running water for 10 minutes and then washed again with distilled water for 5 minutes.

The initial stage of IHC staining is the process of blocking endogenous peroxidase with hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) block solution for 10 minutes and then rinsed with PBS 5 times for 5 minutes each. The next process is dripping protein block solution on the tissue and incubated for 10 minutes and rinsed with PBS 5 times and then given primary antibodies (anti-ER $\alpha$  and PR-A antibodies) at a dilution of 1: 200 each then incubated for 1 hour and rinsed with PBS. For negative control slides, no antibody was applied. The next step is to drip biotinylated goat anti-polyvalent (secondary antibody) and incubated for 10 minutes and then washed with PBS twice. The tissue slides were then dripped with streptavidin peroxidase and incubated for 10 minutes and then washed with PBS twice. The next stage was the administration of diaminobenzine (DAB) chromogen as a visualisation of the staining results for 1-10 minutes while being observed under a microscope. Positive results (immunoreactivity) are marked by the formation of a brown colour with varying degrees of intensity, indicating the presence of antigen and antibody binding in the examined tissue. Then the process of staining the tissue

background (counterstain) using Mayer’s haematoxylin solution. The last stage is the dehydration process, clearing with low to highest concentration alcohol solution (70%, 80%, 90%, and 95%), absolute alcohol (3 repetitions), silol (three repetitions) followed by closing the slide using cover glass (mounting) with Entellan® adhesive material.

The stained slides were observed using a light microscope (Olympus, Japan) equipped with a photographic device (SIGMA) at 100x and 400x magnification. Observation and identification of ERα and PR-A receptors in the uterus were based on the presence of positive reactions visualized by the intensity of the brown color. The identification of staining results was performed using the intensity score (IS) method, as shown in Table 1.

**Table 1:** Intensity scoring method for ERα and PR-A expression in local rabbit uterine tissue.

| Staining intensity  | Score | Description       |
|---------------------|-------|-------------------|
| No immunoreactivity | 0     | No color          |
| Weak expression     | 1     | Light brown color |
| Moderate expression | 2     | Brown color       |
| Strong expression   | 3     | Dark brown color  |

STATISTICAL ANALYSIS

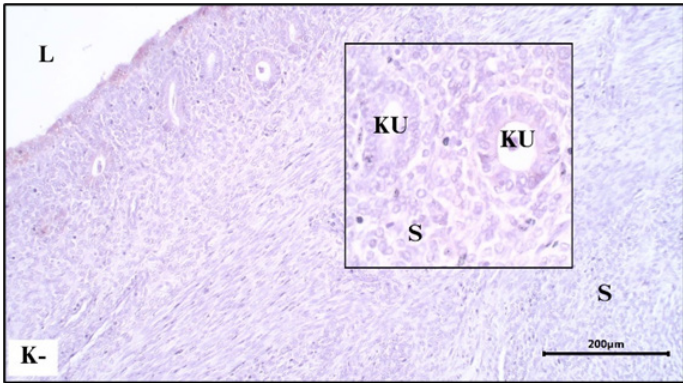
The distribution and expression of ERα and PR-A in the rabbit uterus were analyzed descriptively and presented as histological images. The intensity score (IS) data were analyzed using the Mann-Whitney U test (Sağsöz *et al.*, 2011). with p value = 0.05.

RESULTS AND DISCUSSION

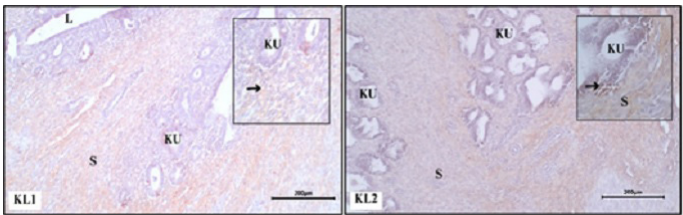
The presence of both hormone receptors (ERα and PR-A) in the uterine tissue of rabbits showed varied distributions. Immunoreactivity visualizations of the presence of ERα and PR-A in the uterus were indicated by the formation of brown coloration in the cell nuclei, connective tissues, and smooth muscle fibers. This indicates the binding between antigens and antibodies used in IHC staining. Positive results for the distribution of ERα and PR-A in the uterine tissues were supported by the absence of expression of both receptors (ERα and PR-A) in the negative control slides used during IHC staining (Figure 1).

In Figures 2 and Figure 3, the distribution of ERα and PR-A in the uterine tissues of local rabbits can be seen, detected in the uterine glands, lamina epithelia, and lamina muscularis. Strong expression of ERα and PR-A was found in the uterus of KL2, while in the uterus of KL1, the distribution and expression were absent. ERα and PR-A were immunoreactive in the uterus of KL2, whereas in KL1, the distribution was minimal and weakly expressed.

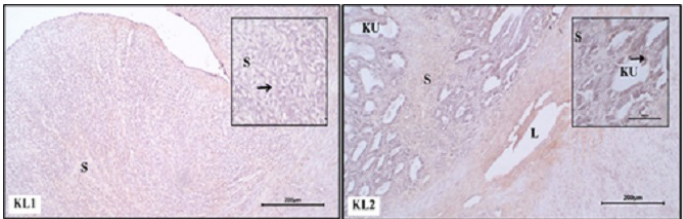
According to Winuthayanon *et al.* (2015), ERα is also expressed in the uterine layer of mice in luminal epithelial cells, myometrium, and stroma. In females, the presence of ERα depends on the estrous cycle phase. In the estrous and proestrous phases, ERα is strongly expressed in luminal epithelial cells (Wang *et al.*, 2000).



**Figure 1:** Negative control slide (K-) of IHC staining on the uterine tissue from the local rabbits. IHC staining was performed using the ABC method. Scale bar: 200 µm.



**Figure 2:** Distribution of estrogen receptor alpha in the uterus of local rabbits KL1 and KL2. ERα expression was shown by brown color as a result of immunoreactivity (arrow). Lumen (L), uterine glands (UG), stroma (S). IHC staining was performed using the ABC method. Scale bar: 200 µm.



**Figure 3:** Distribution of progesterone receptor-A in the uterus of local rabbits KL1 and KL2. PR-A expression is shown by brown color as a result of immunoreactivity (arrow). Lumen (L), uterine glands (UG), stroma (S). IHC staining was performed using the ABC method. Scale bar: 200 µm.

According to Patel *et al.* (2015), progesterone influences uterine function, as evidenced by the presence of PR-A in the stromal cells and lamina epithelia of the uterine endometrium. Progesterone receptors in the uterus can be found in uterine glands, which increase during the luteal

phase (Wu *et al.*, 2018). PR-A receptors decrease on the first and third days of pregnancy, then increase again on the fourth day (Anzaldúa *et al.*, 2007).

Based on the intensity score results, the expression of ERα and PR-A in the uterine tissues of local rabbits in KL1 and KL2 can be seen in Table 2.

**Table 2:** Mean (±SD) intensity score of ERα and PR-A expression in the uterus of local rabbits.

| Expression | Treatment group        |                          |
|------------|------------------------|--------------------------|
|            | KL1 (Control/NaCl)     | KL2 (BPE)                |
| ERα        | 0.56±0.46 <sup>a</sup> | 1.33 ± 0.42 <sup>b</sup> |
| PR-A       | 0.39±0.39 <sup>c</sup> | 1.39 ± 0.25 <sup>d</sup> |

<sup>a,b</sup> Different superscripts in the same row indicate significant differences (P<0.05); <sup>c,d</sup> Different superscripts in the same row indicate highly significant differences (P<0.01).

There was a significant difference (P<0.05) in ERα expression between the two treatment groups in the rabbit uterus, while PR-A expression showed a highly significant difference (P<0.01) between the groups. These results suggest that BPE and hCG induction followed by mating increased progesterone concentrations as the corpus luteum persisted and continued to produce progesterone. The distribution of ERα and PR-A expression in the uterus of the negative control group (KL1) appeared weaker compared to the treated group (KL2) (Figures 2 and Figure 3). ERα expression (P<0.05) and PR-A (P<0.01) showed significant and highly significant differences between the two groups.

Superovulation induction followed by mating stimulates the release of LH from the anterior pituitary, which increases ovulation stimulation. The large number of oocytes ovulated in response to superovulation increases the secretion of oestrogen and progesterone in the ovaries. The secretion of oestrogen and progesterone leads to the growth and development of uterine endometrial tissues. This is in line with Mege *et al.* (2007), who stated that uterine growth and development in pigs could occur due to increased estrogen and progesterone secretion after superovulation treatment using PMSG and hCG.

Immunolocalization of the distribution and expression of ERα and PR-A in the uterine tissues of local rabbits obtained in this study indicates that the BPE induction used in KL2 has potential in inducing superovulation. Progesterone concentration is commonly used to measure the presence of superovulation as it indicates an increase in the number of ovulations or the amount of corpus luteum formation. The increase in PR-A may correlate with an increase in progesterone concentration so that it can be an indicator of successful superovulation. This is in accord-

ance with previous reports that there was an increase in progesterone expression (Syafuruddin *et al.*, 2023) and progesterone concentration (Syafuruddin *et al.*, 2022) in rabbits induced pseudo pregnant with GnRH. The activity of the hormone GnRH is relatively the same as the hormone FSH and LH. However, these results need to be supported by an examination of oestrogen and progesterone concentrations in both groups of rabbits.

CONCLUSIONS AND RECOMMENDATIONS

It is concluded that BPE can increase the expression of ERα and PR-A in the endometrial layer of the local rabbit uterus which can be used as an indicator of successful superovulation. Further research is needed to develop BPE for superovulation methods in other livestock species commonly used for embryo transfer activities.

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NOVELTY STATEMENT

This study is the first to determine the success of superovulation with BPE by observing the expression of estrogen and progesterone receptors.

AUTHOR'S CONTRIBUTIONS

Tongku Nizwan Siregar, Cut Intan Novita and Zahra Shafa Hudzaifa: Conceptualization; Cut Intan Novita, Zahra Shafa Hudzaifa, Tongku Nizwan Siregar, Sri Wahyuni, and Teuku Armansyah: Methodology, formal analysis, and investigation; Zahra Shafa Hudzaifa: data processing; Tongku Nizwan Siregar, Cut Intan Novita, Zahra Shafa Hudzaifa: 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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