Punjab University Journal of Zoology

OPEN ACCESS

37(1): 29-34 (2022)
https://dx.doi.org/10.17582/journal.pujz/2022.37.1.29.34

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Research Article

Superovulation Induction with Bovine Pituitary Extract in Local Rabbits

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Article History
Received: September 22, 2021
Revised: January 03, 2022
Accepted: January 26, 2022
Published: March 17, 2022

Authors' Contributions
AS and TNS planned and executed the work. AS and HH wrote the manuscript. HM helped in experiments. MJ, SS and TN collected the smaples and conducted the experiments. TNS, AS and HH supervised the study. TNS proofread the manuscript.

Keywords
Bovine pituitary extract, Local rabbit, Superovulation, Progesterone, Estrogen

Abstract | The purpose of this study was to analyze the effect of bovine pituitary extract (BPE) on increasing the number of offspring per birth and the concentration of steroids (estrogen and progesterone) in the local rabbits. In this study, we used 10 non pregnant rabbits that have given birth and were healthy clinically. All rabbits were randomly divided into two treatment groups, namely control group (K1) and treatment group (K2) where each group consist of five rabbits. The rabbits of K1 group were injected with aquabides for three consecutive days at a doses of 1.0, 0.5 and 0.3 mL each, whereas rabbits K2 were injected with BPE for three consecutive days at a doses of 1.0, 0.5, and 0.3 mL each. After the third injection, rabbits of K1 and K2 groups were mated with male local rabbits. For measurement of steroid hormones concentration using an enzyme-linked immunosorbent assay (ELISA), blood samples were collected on day 0 (the day when rabbits mated) for estrogen measurement and on day 7 and day 14 for progesterone measurement. Furthermore, pregnancy examination using ultrasound was carried out on the 19th day after rabbits were mated. The average concentration of estrogen on day 0, progesterone concentration on day 7 and 14 of K1 vs K2 gropus were 116.17±10.84 vs 118.46±39.69 pg/mL, 3.72±1.04 vs 10.00±5.30 ng/mL, and 1.01±0.65 vs 11.95±5.38 ng/mL, respectively (P>0.05). Additionaly, the average number of fetuses of K1 and K2 rabbits were 5.20±1,095 and 7.20±2.490, respectively. It can be concluded that the administration of bovine pituitary extract in K2 group tends to increase the concentration of estrogen and progesterone and the number of fetuses in local rabbits.

Novelty Statement | The study is novel as it is a preliminary research on pituitary extract of bovine for superovulation.

To cite this article: Sayuti, A., Hamdan, H., Jannah, M., Safriadi, S., Nasri, T. and Siregar, T.N., 2022. Superovulation induction with bovine pituitary extract in local rabbits. *Punjab Univ. J. Zool.*, **37**(1): 29-34. https://dx.doi.org/10.17582/journal.pujz/2022.37.1.29.34

Introduction

Superovulation and embryo transfer programs can be applied as part of a mating technique aimed at producing large numbers of offspring with superior genetic qualities. Superovulation in rabbits was carried out with the purpose of studying the development of this technology in other species (Garnier *et al.*, 1988). The consideration of the use of rabbits was based on certain advantages that they possess, such as a relatively short reproductive cycle and low maintenance costs (Saratsi *et al.*, 2002).

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All superovulation preparations used were aimed at inducing ovulation in larger quantities. Methods for induction of superovulation in rabbits had been reported with PMSG (Stheshkumar, 2006), hMG (Kanayama *et al.*, 1995), a combination of PMSG with hCG or GnRH (Saratsi *et al.*, 2002; Dorra *et al.*, 2013), and in combination with PGF (Marhaeniyanto dan Kasthama, 2007). Superovulation has been previously studied in New Zealand White and Soviet Chinchilla rabbits, and was carried out using 150 IU PMSG. In these studies, it was found that there was no effect on either breed with regards to the superovulation response (Satheshkumar, 2006).

To date, the most popular method for performing superovulation is through the use of FSH and PMSG. Superovulation using the FSH hormone has a very good response, but because it has a very short half-life of approximately 2-5 hours, the injections need to be preformed repeatedly. PMSG on the other hand has a much longer half-life so that it often causes continuous estrus. Various efforts have been made to overcome the obstacles mentioned above; one of which being the use of pituitary extract to increase animal productivity. The pituitary gland is one of the main sources of the FSH hormone. The bovine pituitary gland contains at least 10 kinds of hormones including follicle-stimulating hormone (FSH), luteinizing hormone (LH), growth hormone (GH), luteotropic hormone (LTH), adrenal corticotropic hormone (ACTH), thyroid somatotropic hormone (TSH), and lipogenic hormones. These hormones are produced by the anterior pituitary lobe. Several recent studies have shown that pituitary extract can increase livestock productivity (Suriansyah *et al.*, 2013; Amiruddin *et al.*, 2014; Zulkarnain *et al.*, 2015; Outang *et al.*, 2017; Iskandar dan Setiaji, 2018). However, information regarding the use of pituitary extracts for the purpose of superovulation is still limited (Arum *et al.*, 2013).

Evaluation of the success of superovulation induction can be done by knowing the success rate of oocytes and embryos (Daader *et al.*, 2003). The success rate of oocytes and embryos can be assessed by measuring concentrations of the hormones estrogen and progesterone by an increase in the number of offspring (Nur *et al.*, 2006), and an increase in the number of corpus luteum (Arum *et al.*, 2013). The hormones estrogen and progesterone are involved in the manifestation of estrus, ovulation, corpus luteum regression, and pregnancy. According to Amiruddin *et al.* (2014) an increase in the number of developing follicles is indicated by high concentrations of estradiol during estrus. Salisbury and Van Demark (1985) stated that with the growth of follicles, the levels of estradiol in the blood will gradually increase because the growing follicles will produce more follicular fluid and estradiol. In addition to estrogen, an increase in the number of ovulations can be indicated by a high concentration of progesterone in the luteal phase. Concentrations of progesterone increased on the 7th day post-insemination, after induction of superovulation with FSH in Aceh cattle by as much as 17.40±5.8 ng/ml. (Amiruddin *et al.*, 2014)

Materials and Methods

Animal

In this present study, 10 non pregnant rabbits from local breeds, aged 1-1.5 years old, with a bodyweight of 2.8-3.0 kg, that have given birth and were healthy clinically were used. All rabbits were randomly divided into two treatment groups, namely control group (K1) and treatment group (K2) where each group consist of five rabbits.

This study used 25 rabbits from local breeds, aged 1-1.5 years old, that were clinically healthy with a bodyweight of 2.8-3.0 kg.

Pituitary extract production

The bovine pituitary gland was collected from the Banda Aceh City Slaughterhouse. The collected bovine pituitary was put in a coolbox and immediately taken to the laboratory for extract preparation. The pituitary extract was prepared according to the method applied by Isnaini dan Suyadi (2004).

Treatment of rabbits

The rabbits of K1 group were injected with aquabides for three consecutive days at a doses of 1.0, 0.5 and 0.3 mL each, whereas rabbits K2 were injected with BPE for three consecutive days at a doses of 1.0, 0.5, and 0.3 mL each. After the third injection, rabbits of K1 and K2 groups were mated with male local rabbits.

Blood collection and preparation

Blood samples were collected on day 0 (the day the rabbits were mated) for estrogen measurement and on day 7 and day 14 after mating for progesterone measurement. Blood samples were collected from the auricular vein of K1 and K2 rabbits using a 1 ml disposable syringe without anticoagulant. Blood samples were allowed to clot at ambient temperature. Subesquently, serum was separated from the whole blood using a centrifuges and then decanted into micro tubes and stored at -20°C until the time of hormone analysis. Hormonal analysis for measurement of estrogen and progesterone concentration was carried out using an enzyme linked immunosorbent assay method. In this study, two kinds of commercial ELISA kit, namely estradiol (EIA-2693) and progesterone (EIA-1561) (ELISA, DRG International Inc., Germany) were used.

Measurement of estradiol and progesterone concentration

Hormones analysis were performing according to the procedure from the manufacturer (ELISA, DRG International Inc., Germany). Concentration of estradiol

and progesterone were measured separately according to the ELISA kit used. Briefly, duplicate 25 µl alliquots of serum sample were assayed with 25 µl alliquots standard solution and also the quality control on each well of ELISA plate coated with monoclonal antibody of estradiol and progesterone. Furthermore, 200 µl of HRP Estradiol or HRP Progesterone were added to each microplate and incubated at room temperature. After incubation, the solution on the plate was removed and washed with a washing solution four times. After washing, microtiterplates were dried by gently patting them with absorbent paper. Next, 100 µl of substrate solution (TMB substrate) was added to each microtiterplate and then incubated at room temperature. After incubation with the substrate solution, the enzymatic reaction was stopped by adding 50 µl of the stop solution (Stop Solution, 0.5 M) onto each microtiterplate. The absorbance value was then read at a wavelength of 450 nm using an ELISA reader (absorbance microtiterplate reader) that had been equipped with the MPM6 program. The reading was taken no later than 10 minutes after the addition of stop solution.

Pregnancy examination

Pregnancy examination was carried out using ultrasound on all rabbits of K1 and K2 groups on the 19th day after rabbits were mated. This examination was required for obtain the number of fetuses during pregnancy in both groups.

Data analysis

The data obtained in this study was analyzed using student t test and the results of analysis presented in table.

Results and Discussion

The average concentrations of estrogen and progesterone, number of fetuses during pregnancy, and number of offspring at birth after superovulation with pituitary extract are presented in Table 1. The concentrations of estrogen immediately after mating (day-0) in K2 tended to be higher than in K1 group, with concentration of 118.46±39.69 and 116.17±10.84 pg/mL respectively, although statistically there was no significant difference (P>0,05).

Based on Table 1, it can be seen that concentrations of estrogen in rabbits that had undergone superovulation with BPE (K2) was higher than K1, although statistically it shows no significant difference (P>0.05) with concentrations (pg/mL) in K1 and K2, namely 116.17±10.84 and 118.46±39.69. This insignificant difference in estrogen concentrations may be caused by several factors, including the repetition of treatment and the time interval for injection of bovine pituitary extract. According to Siregar and Hamdan (2007), one significant factor that needs to be considered in order to achieve optimal superovulation results is treatment repetition. The classic way of FSH treatment is with multiple doses that are given for four consecutive days in both the mornings and evenings. In this study, the repetition of treatment was only carried out for three days with one injection per day. Another factor that may be the cause of low concentrations of estrogen is the low level of the given hormone found in BPE. According to Shahib (2001), the effectiveness of hormones is not only influenced by blood hormone levels but also depends on the state of the receptors on the target cells. In this study, there was no measurement of FSH or LH hormone levels in the pituitary used, so there is a chance that the BPE dose used was still too low.

Table 1: Local rabbit superovulation responses after administration of bovine pituitary extract (BPE).

Parameters	K1 (Physiological NaCl)	K2 (Bovine pituitary extract)
Steroid hormone concentration		
Estrogen on day-0 (pg/mL)	116.17±10.84	118.46±39.69
Progesteron on day-7 (ng/mL)	3.72±1.04	10.00±5.30
Progesteron on day-14 (ng/mL)	1.01±0.65^{a}	11.95±5.38^{b}
Number of fetuses	5.20±1.09	7.20±2.49
Birth weight	86.0±5.4	87.5±2.8

$^{a, b}$ Different superscripts on the same line show significant differences (P<0.05).

Although not significant, BPE administration tends to increase estrogen concentrations. The FSH hormone contained in the injected BPE will stimulate follicular development so that more preovulatory follicles are produced. This has been proven by Siregar *et al.* (2020), that administration of BPE in white rats will increase the development of the number of primary, secondary, and tertiary follicles. The large number of preovulatory follicles greatly affects the high concentrations of estrogen produced. This is in accordance with the opinion of Sugiyatno *et al.* (2001), that the number of follicles that ovulate will increase the amount of estrogen in a serum.

The concentrations of rabbit progesterone showed not significant difference (P>0.05) between K1 and K2 on the 7th day after mating, 3.72±1.04 ng/mL and 10.00±5.30 ng/mL. Although the increase in the concentration of progesterone in rabbits showed no significant difference (P>0.05), BPE administration tended to increase concentrations of progesterone. Progesterone concentrations in both groups were able to indicate the occurrence of pregnancy. The concentration of progesterone in pregnant rabbits was 3.1 ng/mL (Kleden *et al.*, 2017) or 9.4 ng/ml (Szendro *et al.*, 2010).

Maertens and Luzi (1995) revealed that BPE causes an increase in the number of follicles that ovulate.

After ovulation the cavity left by the splitting of the follicle begins to fill with blood, and the blood forms a structure called the hemorrhagic corpus. As a result of the luteinization process of the hemorrhagic corpus by the influence of the LH hormone from the anterior pituitary gland, the hemorrhagic corpus begins to turn into yellow luteal tissue called the corpus luteum or corpus luteum, which produces the hormone progesterone. The number of corpus luteum is positively correlated with the amount of progesterone present.

The concentrations of progesterone between groups K1 and K2 showed a significant difference (P<0.05) on day 14 after mating which were 1.01±0.65 and 11.95±5.38 ng/mL, respectively. This increase in progesterone was the effect of increasing the number of ovulations, so it can be concluded that BPE is able to increase the number of ovulations in rabbits. Tjitosumirat (2009) reported that the concentration of progesterone can be used to predict the number of ovulations in small ruminant animals.

The decrease in progesterone in the K1 group on day 14 compared to day 7 (1.01±0.65 vs 3.72±4.04) may be related to the occurrence of embryonic death, lysis of the corpus luteum, failure of implantation, or pseudo-pregnancy. Wijono (1998) reported that the highest progesterone levels can occur in the pregnancy phase with maximal corpus luteum development. When pregnancy does not occur or the embryo is not implanted, it is followed by a gradual decrease in progesterone levels caused by the regression of the corpus luteum. Browning *et al.* (1980) reported that concentrations of progesterone in pseudo-pregnant local rabbits was lower than in pregnant rabbits. Progesterone concentrations in pseudo-pregnant rabbits will increase from days 4-5 after induction and decrease to basal concentrations on days 19-20 (Caillol *et al.*, 1983). It is possible that in the local rabbits the decrease in progesterone concentrations started on the 14th day.

In the K2 group, progesterone concentration on day 14 increased compared to day 7 (11.95±5.38 vs 10.00±5.30 ng/mL). This shows that the concentration of progesterone in mid-pregnancy increased because rabbits are included in the corpus luteum dependent species; which are species that depend on progesterone produced by the corpus luteum for the maintenance of pregnancy. According to Arimbawa *et al.* (2012), the increase and decrease of progesterone levels is in line with the development of the corpus luteum.

Progesterone concentrations in blood serum are often used to predict CL function in domestic animals during the luteal phase (Scaramuzzi *et al.*, 1993). In line with the increase in progesterone in the K2 group, treatment with BPE also resulted in a higher number of fetuses during pregnancy compared to the control group (K1) with the number of each in K1 vs. K2 groups being 5.20±1.09 vs 7.20±2.49. Although statistically there was no significant difference (P>0.05), administration of BPE in rabbits tended to increase the number of fetuses. Hafizuddin *et al.* (2010), proved that BPE and PMSG have the same effectiveness in stimulating superovulation in mice. This is probably because the effect of superovulation with BPE is the same as the mechanism of action of superovulation with PMSG. Marhaeniyanto dan Kasthama (2007) stated that rabbits that undergo superovulation with PMSG show a significant increase in the number of embryos they produce. Administering BPE to rabbits in this study increased the number of offspring per birth when compared to normal births, which is 2-4 rabbits per rabbit. This increase in the number of offspring per birth is due to pituitary induction leading to an increase in the number of developing follicles and ovulation. Siregar *et al.* (2020) added that administration of BPE in white mice caused an increase in the number of ovulations even though the number of ovulations was not as superior as that of PMSG induction. The number of ovulations in white mice induced with PMSG and hCG was 9.2±4.32 vs 4.4±3.51, respectively. Arum *et al.* (2013) succeeded in increasing ovulation in Aceh cattle by administering pituitary extract with an average CL amount of 4.

Data on the birth weight of rabbits after superovulation with pituitary extract can be seen in Table 1. The birth weight of rabbits showed an insignificant difference (P>0.05) between K1 and K2 groups, each of which was 86.0±5.4 and 87.5±2.8 g. Although the birth weight of rabbits showed an insignificant difference (P> 0.05), giving BPE to rabbits tended to increase the average birth weight because BPE contains Growth Hormone (GH) which promotes fetal development. Peraita *et al.* (2014) reported that there was an increase in birth weight in pregnant ewes injected with GH. In this study, rabbit pregnancy was detected from the 7th to the 14th day after mating. The data used to determine the number of fetuses was taken from the 14th day post-mating, because the structure and picture of the fetuses were more clearly visible on the ultrasound monitor that day. This reflects the findings of research conducted by Lebas *et al.* (1986) who reported that palpation in rabbits is effective between 10-14 days, and is not effective if done before 9 days after the date of mating. They also reported that there is a risk of abortion occurring if palpation is done after 14 days. In this study, it was concluded that the administration of BPE tended to increase the number of fetuses in pregnant local rabbits, as well as the concentrations of estrogen and progesterone in these rabbits.

Conflict of interests

The authors have declared no conflict of interest.

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