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RESEARCH NOTE

HUMAN CHORIONIC GONADOTROPIN FROM URINE OF PREGNANT WOMEN FOR *IN VITRO* MATURATION OF MADURA CATTLE OOCYTES

Herry Agoes Hermadi[1], Raden Tatang Santanu Adikara[2], Mas'ud Hariadi[1] and Erma Safitri*[1]

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ABSTRACT

The purpose of this study was to test whether human chorionic gonadotropin (hCG) from urine of pregnant women can be used for *in vitro* maturation of Madura cattle oocytes. Urine samples were collected from 50 healthy pregnant women gestating for 1.5 to 3.5 months. Molecular weights of hCG were 37 kDa and 22 kDa. HCG levels measured via ELISA had 10 times the deposition, with an average of 27,333 mIU/l at 1.5 months and 105,667 mIU/l at 3.5 months, respectively. T-test showed no significant difference in oocyte maturation rate between the control group with patent hCG and hCG from pregnant women at P>0.05. This study illustrates that hCG from pregnant women at 1.5 to 3.5 months can aid in *in vitro* maturationof Madura cattle oocytes.

Key words: hCG, *in vitro* maturation, Madura beef cattle, oocytes, urine of pregnant women

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INTRODUCTION

Pregnancy tests, quantitative blood tests and the most sensitive urine tests in humans usually detect hCG between 6 and 12 days after ovulation (Wilcox *et al*., 1999). However, it should be considered that the total hCG level varies a lot within the first four weeks of pregnancy, which makes tests susceptible to error (Butler *et al*., 2001). A 35% increase in hCG level over 48 h is the proposed minimum increment, consistent with an appropriate intrauterine pregnancy test (Kirk *et al*., 2013).

Human chorionic gonadotropin (hCG) is not specific-species and not limited to human hormones, *i.e.,* urine from pregnant women also holds therapeutic effects for receptive animals. In fact, Santibañez *et al*. (2014) suggested that intrauterine injection effect of hCG may be directly involved in *in vitro* fertilization (IVF) process in humans before embryo transfer. HCG is a hormone the placenta produces after implantation (Gregory and Finlay, 1999; Cole, 2009) and this can be detected in several pregnancy tests.

Human chorionic gonadotropin is a glycoprotein composed of 237 amino acids. The molecular mass of intact, α(alpha) and β(beta) hCG are 36.7, 14.5 and 22.2 kDa, respectively (Gam and Latiff, 2005). It is heterodimeric with alpha subunits identical to luteinizing hormone (LH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH) and beta subunit unique to hCG. The α subunit contains 92 amino acids, while β subunit contains 145 amino acids, encoded by six high homologous genes composed of tandems and pairs on chromosome 19q13.3-CGB (Steel and Torrie, 1960; Canfield *et al*.; 1987; Agrawal *et al*., 2000).

HCG treatment, in terms of embryonic

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development and oocyte collection, has been shown to be a more promising treatment to female infertility compared with recombinant human FSH. In the study by Ng *et al.* (2001), 85% of the oocytes reached metaphase at IVF treatment 2; consequently, it was tested whether there would be an additional benefit to administering FSH on top of hCG in priming *in vitro* maturation (IVM). Third to fifth cycles were treated with 75 IU rFSH for 6 days, while 33 cycles were given 10,000 IU hCG 36 h before oocyte retrieval and fertilization via IVF and ICSI. The resulting embryo was replaced on day 2 or 3. A total of 1528 immature oocytes were found. The overall maturation and fertilization rates were 74.2 and 72.8%, respectively. After embryo transfer, 23 pregnancies (33.8%).

The use of hCG in animals, specifically on follicular growth and development *in vivo* or *in vitro*, has been investigated. Due to its similarity to LH, hCG may also be used clinically to induce ovulation in the ovaries. For this reason, some organizations collect urine from pregnant women to extract hCG. Using hCG, comparative follicles begin to occur simultaneously, but many cases become atretic. During the luteal phase of the follicular growth cycle, one of the follicles becomes dominant. Notably, biochemical changes in the development of subordinate follicles to dominant follicles, when analyzed for chemical changes, are different from those in FSH, LH and receptor (Zemitis *et al.*, 2015).

In the study of molecular hCG, folliculogenesis is highly dependent on the influence of gonadotropin hormone; antral folliculi is responsible for FSH-LH. Inhibin, activin, insulin-like growth factor I (IGF I) and protein walls (bonds with proteins) directly affect granulosa cells and techa cells that stimulate follicular growth and steroidogenesis (Gardner *et al.*, 2004). Injection of 1,000 IU hCG exogenously within 24 h of sow postpartum farrowing induced ovulation in 41%-75% of the population at 7-10 days after injection (Armstrong *et al.*, 1999; Kirkwood *et al.*, 1999). Postpartum sow ovaries have potentially estrogenic medium-sized follicles (4 to 5 mm), and some sows have exhibited estrous behavior (De Rensis *et al.*, 1993; Sesti and Britt, 1993; Sesti and Britt, 1994; Langendijk *et al.*, 2007). But the postpartum estrous behavior observed at 2=4 days postpartum farrowing is anovulatory, which is likely due to the inability to generate a pre-ovulatory LH surge (Sesti and Britt, 1993).

Medical experts up to this day have been relying on LH activity-dependent alone in inducing final oocyte maturation, which overlooks the redundancy of the natural waves of mid-cycle FSH surge. Thus, this needs to be resolved. Evidence from clinical research suggests that in an undetermined subset of patients, the LH and FSH waves are doubly advantageous compared to the LH spike in the form of a human chorionic gonadotropin (hCG) trigger. Double waves can be triggered by gonadotropin-releasing hormones, agonist boluses, causing endogenous LH and FSH flare-ups and resembling natural waves of concomitant hCG to ensure adequate exposure to LH activity (Kol and Humaidan, 2010). Further research is needed to characterize hCG from urine of pregnant women, which can then be potentially used for *in vitro* maturation of Madura cattle oocytes.

The purpose of this research was to extract hCG from urine of pregnant women gestating at 1.5 to 3.5 months, characterize and determine the molecular weight of the hCG protein and perform a biological potential test to determine its effect on the maturation level of Madura cattle oocytes.

MATERIALS AND METHODS

HCG isolation, identification and purification

Urine samples were taken from fifty healthy pregnant women gestating for 1.5 to 3.5 months. Samples were obtained by a midwife from a clinic in Surabaya, East Java, Indonesia. In the morning, 100 ml of urine was collected from each pregnant woman and these were centrifuged at 3,000 rpm for 15 min at 4°C to separate the metabolite cells. Thirty mg/100 ml activated charcoal powder was added, and the mixture was centrifuged at 3,000 rpm at 4°C for 20 min (Green and Leake, 1987) until mixture was homogeneous. Charcoal removes toxic substances, dyes and steroid hormones; it also absorbs and inactivates organic chemicals. Supernatant was filtered through an Erlenmeyer tube using a filter paper, producing a 50 ml supernatant. This was poured into Sephadex G-100 filtration device and poured into a chamber with running buffer

to carry out electrophoresis (Sakakibara *et al.*, 1987). Furthermore, hCG extract obtained from urine extract was processed through SDS-PAGE and ELISA (Gam and Latiff, 2005). A running gel was inserted through the wall roughly less than the upper limit. Around 1 ml of butanol was added, and the gel was left for 25 min until it has solidified. Butanol was discarded, and the gel was cleansed with PBS and dried with Whatman paper. Comb was removed and remnants of the gel with buffer were cleaned.

HCG concentration

HCG concentration was determined through ELISA based on the sandwich principle (Mahaputra and Mustafa, 2003). The microtiter wells were coated with a monoclonal antibody directed towards a unique antigenic site of the hCG molecule. An aliquot of sample containing endogenous hCG was incubated in the coated well. After washing, a second incubation followed with an enzyme conjugate, an anti-hCG antibody conjugated with horseradish peroxidase. After incubation, the unbound conjugate was washed off. The amount of bound peroxidase was proportional to the concentration of hCG in the sample. Having added the substrate solution, color intensity developed was proportional to the concentration of hCG in the sample (Kariman *et al.*, 2011).

***In vitro* maturation of cattle oocytes**

Extracted hCG was used to investigate its potential in inducing *in vitro* maturation in Madura beef cattle oocytes. There were two setups: the control group was given TCM 199 + 0.5 IU pregnant mare serum gonadotrophin (PMSG) + 0.5 IU hCG (Chorulon, Intervet, Holland), while the experimental group received the same treatment but with 0.5 IU hCG from gestating women.

Ovaries from slaughterhouses were washed 2 to 3 times with physiological saline solution, placed in a glass above water bath at 37°C, taken one by one with sterile tweezers and dried with sterile tissue paper. Using a 10 ml sterile disposable syringe, samples were washed with 1.5 ml of oocyte washing medium. Suction was done by stabbing the adjacent part of the ovary parenchyma in the next follicle bubble and directing the needle tip to the follicle (approximately 5 mm in diameter) near the point of needling (without removing the needle first). After each follicle fluid was siphoned off (about 3-4 ml), this was carefully transferred into test tubes to avoid mechanical damage to the oocyte and placed in a water bath. After the oocyte had dropped to the bottom of the tube, precipitate was evaluated by placing it on a large petri dish and examining under a stereo microscope (Mahaputra and Mustofa, 2003).

Once an oocyte was found, this was collected with a modified pastry pipette (of the same diameter as the oocyte), placed on a smaller petri dish with washing medium and examined again under a microscope to determine its quality (Mahaputra and Mustafa, 2003). Afterwards, all oocytes were washed 3-4 times with washing medium and was finally washed with 2.5-3 ml of tissue culture medium 199 (TCM 199). Oocytes with no cumulus debris were transferred into the maturation medium, dropping 100 µl each in a petri dish. A total of 10 oocytes were used in this study, prepared 2 h earlier in incubator with 5% CO_2. Four 100 µl drops of maturation medium were placed in each 35 mm sterile petri dish. Each drop of medium can culture as much as 5 oocytes. Media were then covered with mineral oil and placed in an incubator containing 5% CO_2 set at 39°C and humidity of 95-100% for 24 h. One percent aceto-orcein stain was added to determine maturity level of egg cell (Chen *et al.*, 2013).

To collect data on egg maturity, harvested eggs were placed and covered on the glass object, then placed in a fixative solution for 48 h. These were stained with 1% aceto-orcein for 2-3 min, washed with solution and viewed under an inverted microscope. Assessment of egg maturity level was based on germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I and metaphase II criteria (de Oliviera *et al.*, 2016). Mature oocytes were identified by locating those which can resume meiosis (extruding a first polar body) and can undergo fertilization (Fig. 1).

Data analysis

Data on identification, isolation, purification and measurement of hCG concentration from the urine of pregnant women at 1.5 to 3.5 months were processed descriptively. Student's t-test (Steel and Torrie, 1960) was used to determine the difference on oocyte maturation rate between patent hCG (Chorulon, Intervet, Holland) and

hCG from urine of pregnant women.

RESULTS AND DISCUSSION

HCG isolation, identification and purification

SDS-PAGE results showed that hCG from the urine of pregnant women had molecular sizes of 37 kDa and 22 kDa (Fig. 2). Gam and Latiff (2005) detected other bands from non-reduced fractionally desialylated-hCG sample: 43.5, 38.5, 29.45 and 20.85 kDa, which, after being reduced, resulted to an uncompounded pledge at 35.2 kDa.

Calculation of hCG concentration

The human pregnant urine ammonium sulphate precipitated concentrations of hCG at age 1.5 and 3.5 month were 27,333 mIU/l and 105,66 mIU/l, respectively. The measurement of hCG provides a specific test for pregnancy. Lyophilized hCG, although stable at room temperature for 3 weeks, should be stored at 2-8°C (Suthar and Shah, 2009). It is advised to reconstitute it in sterile 18 M-cm H_2O at a concentration of 1,000 IU/ml, which can be further diluted to other aqueous solutions.

In vitro maturation of cattle oocytes

Oocytes that matured *in vitro* are capable of resuming meiosis, extruding a first polar body and can undergo fertilization (Fig. 1). In this research, the rate of maturation of cow oocytes in TCM 199 + 0.5 IU PMSG + 0.5 IU hCG (Chorulon, Intervet, Holland) was 91.15± 8.57, while maturation rate in TCM 199 + 0.5IU PMSG + 0.5 IU hCG from pregnant women was 90.54±12.86. T-test showed no significant difference between groups (P>0.05) (Table), suggesting that hCG from pregnant woman can be used for maturation process of cow oocytes.

The dairy cattle industry has perfected the application of the first reproductive biotechnology, *i.e.*, artificial insemination (AI), a success story which made use of embryo transfer technology (ETT). In addition, emerging researchers have taken interest in the field of transvaginal oocyte recovery (TVOR) and *in vitro* embryo production (IVEP). IVF has paved the starting point for the production of generative material for many sophisticated reproduction techniques, like sperm microinjection into mature oocytes. In several countries, commercial IVF facilities are already being serviced by cattle ET operators. Also, various research groups have reported on the modification of TVOR (Suthar and Shah, 2009).

This study tested the use of hCG from urine of pregnant women in inducing maturation of cattle oocytes. Recorded molecular weight of hCG via SDS-PAGE was 37 and 22 kDa. HCG concentrations via ELISA assay had 10 times the deposition, with average level of 27,333 mIU/l at 1.5 months and 105,667 mIU/l at 3.5 months. T-test revealed no significant difference (P>0.05) between groups. This study illustrates that hCG extracted from urine of pregnant women at 1.5 to 3.5 months can be used to induce *in vitro* maturation in Madura cattle oocytes.

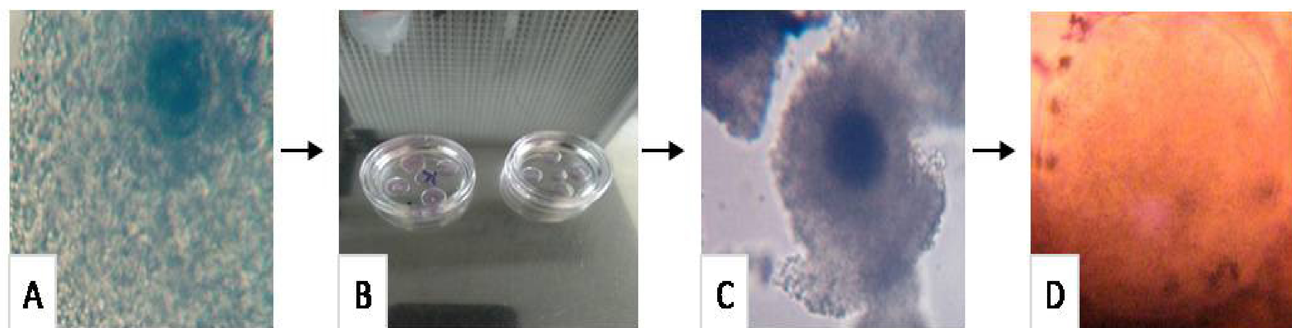

Fig. 1. *In vitro* maturation culture of Madura beef cattle oocytes at different stages. A: addition of hCG to maturation culture; B: immature oocyte; C mature oocyte; D: egg cells at metaphase stage.

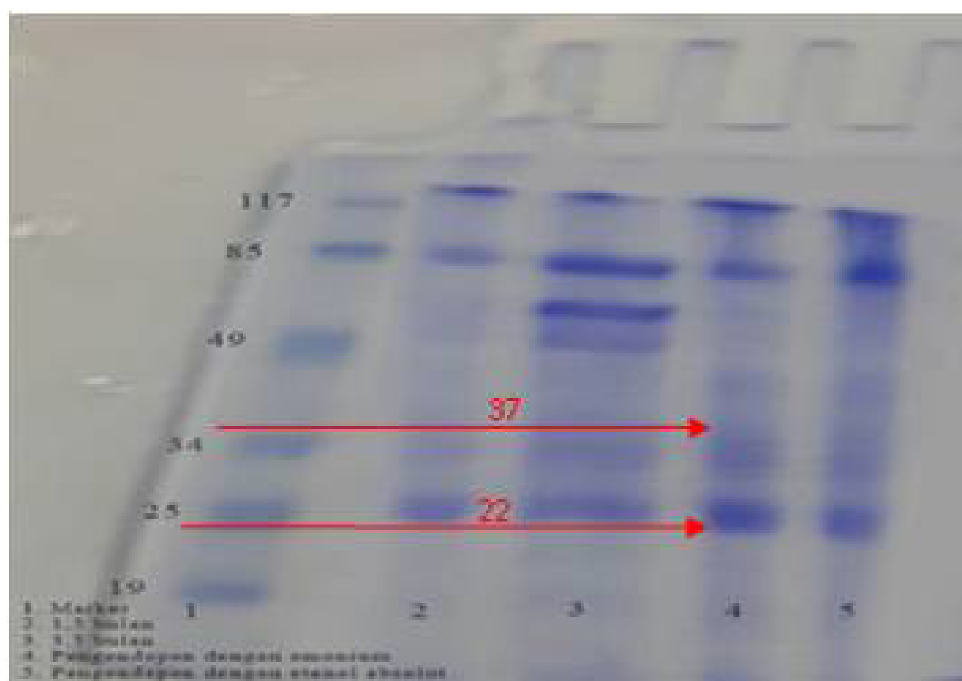

Fig. 2. SDS-PAGE shows molecular weight of hCG from urine of pregnant woman at 37 and 22 kDa.

Table. Rate of maturation in cattle oocytes induced by different HCG sources.

hCG used	*n*	Mean ±SD
TCM 199 + 0.05 PMSG + 0.5 IU hCG 0.5 hCG (Chorulon, Intervet, Holland)	50	91.15± 8.57[a]
TCM 199 + 0.5 IU PMSG + 0.5 IU hCG from pregnant women	50	90.54±12.86[a]

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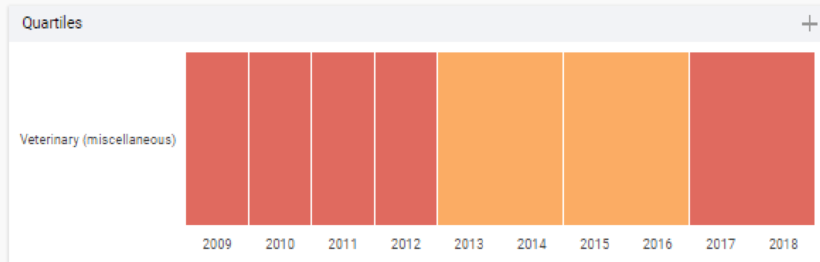

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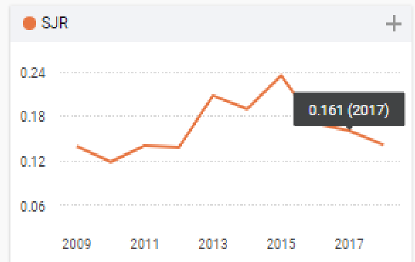

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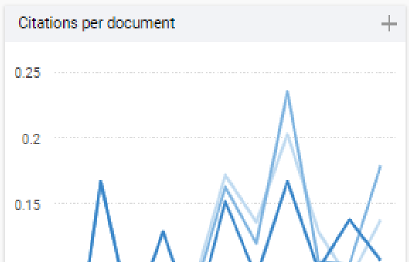

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