Research Article

Epidermal Growth Factor (EGF) and Basic Fibroblast Growth Factor (FGF2) Profiles in Follicular Fluid, Maturation Media and Embryo Culture of Bali Cattle

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Abstract | A study on growth factors in Bali cattle embryo culture media *in vitro* has never been carried out and this study aims to measure the concentration of FGF2 and EGF in follicular fluid, maturation media and Bali cattle embryo culture media. The study used ovaries that came from slaughterhouse and were oocytes mature before fertilization using semen from one bull, oocytes are fertilized then cultured for 48 hours. Embryo grouping based on cell count was conducted after 48 hours of culture and was recultured for 48 hours. Follicular fluid, oocyte maturation media and embryo culture media were collected using a disposable syringe and centrifuged, then supernatant in the collection, stored and frozen with -20 °C and growth factor concentrations were measured using the ELISA method. The results showed that there were 228 developing embryos (41%) after 48-hour of culture with 2-, 4-, 8-, and 16-cell division. EGF concentrations in the medium of 2-cell to 16-cell ranged from 0.8539 ng/mL-0.2838 ng/mL. The concentration of FGF2 in follicular fluid is 0.629 ng/mL, while the concentration of FGF2 in maturation media, culture media 2 cells, 4 cells, 8 cells and 16 cells respectively, namely 0.032 ng/mL, 0.015 ng/mL, 0.011 ng/mL, 0.009 ng/mL and 0.008 ng/mL. In conclusion, EGF concentration was found more in follicular fluid and culture media and was not found in maturation media. while FGF2 was found to be higher in follicular fluid than in maturation media and embryo culture media. The EGF and FGF2 concentration decreases as the embryonic development stage increases.

Keywords | Bali cattle embryos, Basic fibroblast growth factor (FGF2), Epidermal growth factor (EGF), *In vitro* culture

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INTRODUCTION

The production of Bali cattle embryos *in vitro* still needs to be improved, especially the quality and quantity of embryos that are worthy of transfers. The development of the culture system in embryos is important in supporting the successful production of embryos. A big challenge for *in vitro* production laboratories is to increase the number of embryos produced with good quality at each cycle of *in vitro* culture (Abd El-Aziz et al., 2016). Factors that affect the development of embryos produced *in vitro* include the quality of oocytes, semen, the medium used and the condition of the culture. Various efforts have been made to improve the quality of the embryo produced, one of

which is the addition of growth factor (GF) to the culture media used (Zahmatkesh et al., 2013). The Growth Factor in cattle includes Epidermal Growth Factor (EGF) and Basic Fibroblast Growth Factor (FGF2).

EGF consists of a single polypeptide chain of 53 amino acids that displays three internal disulfide bonds. Epidermal Growth Factor (EGF) stimulates cell growth, proliferation and differentiation in various tissues by binding to its receptors (Epidermal Growth Factor receptor/EGFR) (Luo et al., 2020). In the ovaries, EGF exerts a strong regulatory effect on the proliferation and differentiation of granulose cells (Zhang et al., 2010). Richani and Gilchrist (2018) showed that EGF kinase receptors contribute to the resumption meiosis induced by luteinizing hormone (LH) from oocytes through gap junction closure and a decrease in cyclic Guanosine Monophosphate (cGMP) follicles. The released EGF binds the EGF receptors located in the cumulus cells (He et al., 2021). Part of the EGF receptor subfamily, the Receptor Tyrosine Kinase-3 (ErbB3), was expressed at the embryonic 2-cell stage and bovine blastocyst (Yoshida et al., 1998).

FGF has an important role in folliculogenesis, including activation of granulosa cell regulation, cumulus cell mitosis and glycolysis processes in primordial follicles (Sugiura et al., 2008). FGF acts as a mitogen, morphogenic, and angiogenic factor to regulate the early development of embryogenesis (Schams et al., 2009). FGF2 is involved in the early development of the human reproductive tract and takes part in the suppression of spontaneous onset of apoptosis as well as being associated with progesterone's ability to maintain the survival of granulose cells. In addition to granulose cells, FGF2 also inhibits apoptosis cell (Kurowska et al., 2020). FGF2 can be identified on all components of the ovary including granulosa cells, oocytes, the basal membrane of the follicle and part of the surface of epithelial cells (Berisha et al., 2004). It has been found that FGF2 acts as an angiogenic factor that stimulates steroidogenesis in granulose cells and increases the activity of tissue plasminogen activators (tPA), thereby increasing oocyte maturation; in addition, FGF2 helps steroidogenesis processes and improves granulosa cell survival (Mishra et al., 2016). FGF signaling through FGFR plays an important role in the development and maintenance of mature vascular tissue in the embryo (Xie et al., 2020).

Growth factor measurements have been carried out on stem cell culture media (Noverina et al., 2019), but have never been conducted in Bali cattle embryos cultured *in vitro*. Currently, information about the role of Growth Factor (EGF and FGF2) in stimulating cell division in embryo production *in vitro*, and their concentration in follicular fluid and culture media is still very limited or has not been analyzed. Therefore, research on EGF and FGF2 was conducted on Bali cattle embryo cultured *in vitro*.

MATERIALS AND METHODS

Oocyte collection and selection

A total of 105 pairs of ovaries with 556 selected oocytes were used in this research. The cattle ovaries were obtained from slaughterhouse and were brought to laboratories with transport media (0.9% NaCl solution, Sigma-Aldrich, USA) added with gentamicin (Sigma-Aldrich, USA). The oocytes collection was carried out by using the slicing method. Furthermore, those oocytes were selected using the Olympus SZ51 microscope 40× magnification, Japan.

In vitro maturation

The selected oocytes were then washed using phosphate buffered saline (PBS) (Gibco by life technologies, USA), added with 0.2% bovine serum albumin (BSA) (Sigma-Aldrich, USA), were matured in a maturation medium consisting of M199 (Gibco by life technologies, USA), added with 0.3% BSA, 10 IU/mL Pregnant Mare Serum Gonadotrophin (PMSG) (Intergonan, Intervet Deutschland GmbH), 10 IU/mL human chorionic gonadotrophin (hCG) (Chorulon, Intervet international BV Boxmeer-Holland), and 50 µg/mL gentamicin (Sigma-Aldrich, USA). Maturation was carried out in the form of droplet (80 µL/drop) and was covered with mineral oil (Sigma Chemical Co. St. Louis MO, USA). Maturation was performed in a 5% CO_2 incubator with at 38.5 °C for 24 hours (Hasbi et al., 2017).

In vitro fertilization

The semen used was collected from the same bull, thawed for 20 minutes at a temperature of 37 °C, and centrifuged for 5 minutes at a speed of 1500 rpm twice. Then, the thinning media and spermatozoa were separated, and media was added to the semen so that the final concentration of spermatozoa was 1.5 x 10^6 cells/mL (Hasbi et al., 2020). After that, four drops were placed on the petri dish (80µL/drop) which was closed using mineral oil (Sigma Chemical Co. St. Louis MO, USA) and calibrated for 30 minutes. Then the oocytes from maturation were put into *in vitro* fertilization (IVF) media (Suzuki et al., 2000) and stored in an incubator with at 38.5 °C and a CO_2 concentration of 5% for 5-6 hours.

In vitro culture

After 5-6 hours of fertilization the oocytes were washed twice using culture medium, transferred in a drop of 80 µL CR1aa culture medium (modification by Sagirkaya et al., 2006) added with 5 mg/mL of BSA, 2.5% FBS and covered with mineral oil (Sigma Chemical Co. St. Louis MO, USA), which further was cultured in an incubator at

38.5 °C and a CO_2 concentration of 5% (Zahmatkesh et al., 2013). Evaluation of the embryo division ability was carried out 48 hours after the next culture. The developing embryos were grouped based on the stages of division (2-, 4-, 8-, and 16-cell). Embryos that had been grouped were recultured for 48 hours used the new media and reevaluated and carried out a collection of culture media from each group of cells.

Media collection

Follicular fluid was collected by employing aspiration method with disposable syringe. The follicular fluid collected was centrifuged for 20 minutes at 1000g, and supernatant collected was stored and frozen at -20 °C. Maturation media and culture media of embryo were collected after reculturation for 48 hours, and was centrifuged for 20 minutes at 1000g with the same procedure as that of follicular fluid.

Growth factor measurement method

Measurement was carried out using enzyme-linked immune sorbent assay (ELISA) KIT for follicular fluid samples, maturation media and culture media of embryo.

Epidermal growth factor (EGF) ELK9094

EGF measurements on the medium were carried out based on the research conducted by Widowati et al. (2016) and Noverina et al. (2019) with several modifications, such as Well was prepared, 100 µL was added to each standard working solution and the sample was then incubated for 80 minutes at 37 °C. The solution was aspirated and washed with 200 µL 1× Wash Solution on each well and was let stand for 1-2 minutes. The remaining was washed 3 times. 100 µL working solution Biotinylated Antibodies was added and incubated for 50 minutes at 37 °C, inspired and washed 3 times. 100 µL of Streptavidin-HRP working solution was added to each well and incubated for 50 minutes at 37 °C. It was then aspired and washed 5 times. A total of 90 µL of TMB substrate solution was added to each well and incubated for 20 minutes at 37 °C (protected from light). 50 µL Stop reagent was added to each well. Absorbance was measured at 450 nm using ELISA reader (Multiskan Go, Thermo Scientific, AS).

Fibroblast growth factor 2 (FGF2) (ELK546)

The measurement of FGF2 on the medium was carried out based on the research conducted by Widowati et al. (2017) and Noverina et al. (2019) with several modifications prepared 50 µL standards or samples were added to each Well. 50µL Biotinylated-Conjugate (1x) was added to each well, mixed and incubated for 1 hour at 37 °C. It was then aspired and washed three times. Each Well was washed and filled with Wash Buffer (250 µL). 100 µL Streptavidin-HRP (1x) was added to each Well and closed, then was incubated for 1 hour at 37 °C. The Well was aspired and washed 5 times with Wash Buffer (250 µL). 90 µL substrate solution was added to each Well, incubated for 20 minutes at 37 °C (protected from light). 50 µL stop solution was added to each Well. The absorbance was measured at 450 nm using ELISA reader (Multiskan Go, Thermo Scientific, AS).

Statistical analysis

Data on embryonic development and Growth Factor concentration were analyzed descriptively. Determination of the relationship between growth factor concentration and percentage of embryos reached morula after reculture using correlation test was conducted with SPSS 20.00.

RESULTS AND DISCUSSION

The embryonic development rate of Bali cattle 48 hours after culture was 41.01% (Table 1). The embryonic development ability of Bali cattle after 48 hours of culture consists of 2-, 4-, 8- and 16-cell. Embryonic cell morphology in Bali cattle cultured for 48 hours can be seen in Figure 1. Embryonic development that has been grouped by cell group and re-cultured 48 hours showed that some embryos are unable to develop further and remain with the same number of cells. The group of 2 cells has not been able to reach morula and in groups 4, 8, and 16 cells were able to achieve morula respectively 13.16%, 29.03%, and 48.28%.

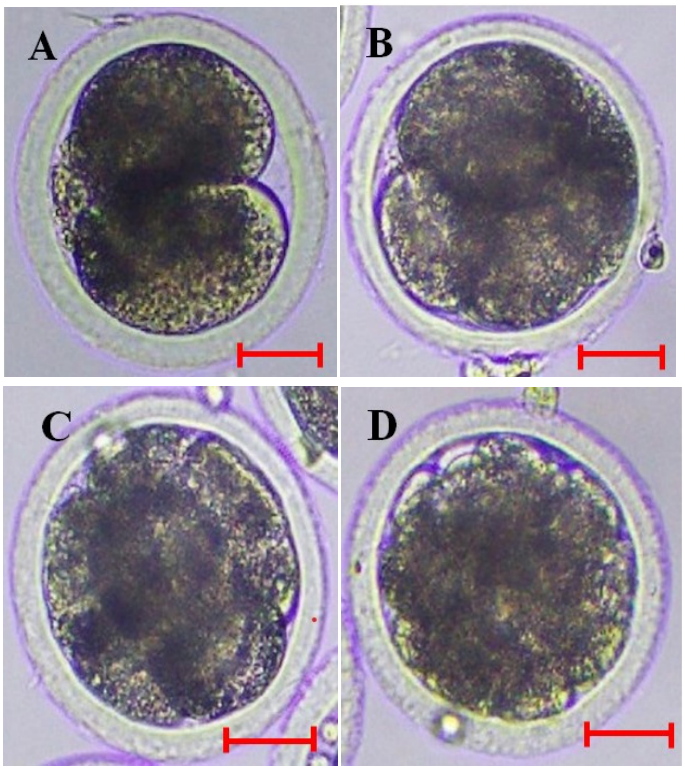

Figure 1: Embryonic development of Bali cattle, (A) 2 cells, (B) 4 cells, (C) 8 cells and (D)16 cells. Scale bars (40 µm).

Tabel 1: The Development of Embryo Bali Cattle Early Culture 48 Hpi

Description	Culture (48 hpi)
Number of ovaries (Pair)	105
Number of oocytes	556
Development rate (%)	228 (41.01)*
2 cells	70/228 (30.70)
4 cells	68/228 (29.82)
8 cells	61/228 (26.75)
16 cells	29/228 (12.72)

Hpi, hours post-insemination. * number of embryos recultured.

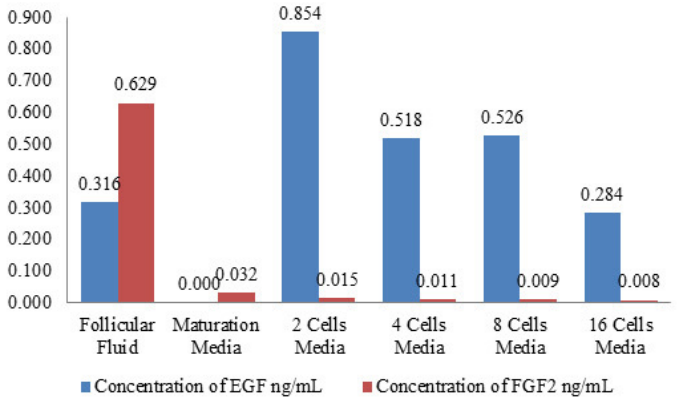

Figure 2: EGF and FGF2 Profile on Follicular fluid, maturation media and culture media of Bali cattle.

The concentration of EGF and FGF2 can be seen in Figure 2. Based on the results of this study, EGF was found in follicular fluid and culture media, but in maturation media it was not found. The concentration of EGF in follicle fluid is 0.32 ng/mL and culture media 2 cells to media 16 cells, which ranges from 0.85-0.25 ng/mL. Research shows the possibility of EGF being produced is so little at the time of *in vitro* maturation that it is not expelled on the maturation medium. However, EGF is needed more for embryonic development so that in the culture medium a higher concentration is found. Higher FGF2 concentrations were found in follicle fluid than in maturation media and culture media. The results of this study found that FGF2 may have a very important role with the development of oocytes in the follicles.

The correlation relationship of embryos reached morula after re-culture with EGF and FGF2 concentrations can be seen in Figure 3. The correlation value of the concentration of EGF and FGF2 values with the percentage of embryos reached morula after reculture, namely -0.8754 and -0.7920, means that the embryo's ability to develop with EGF and FGF2 concentrations has a strong relationship. EGF and FGF2 concentrations decreased with increasing embryonic developmental stage, indicating a negative correlation (Figure 3).

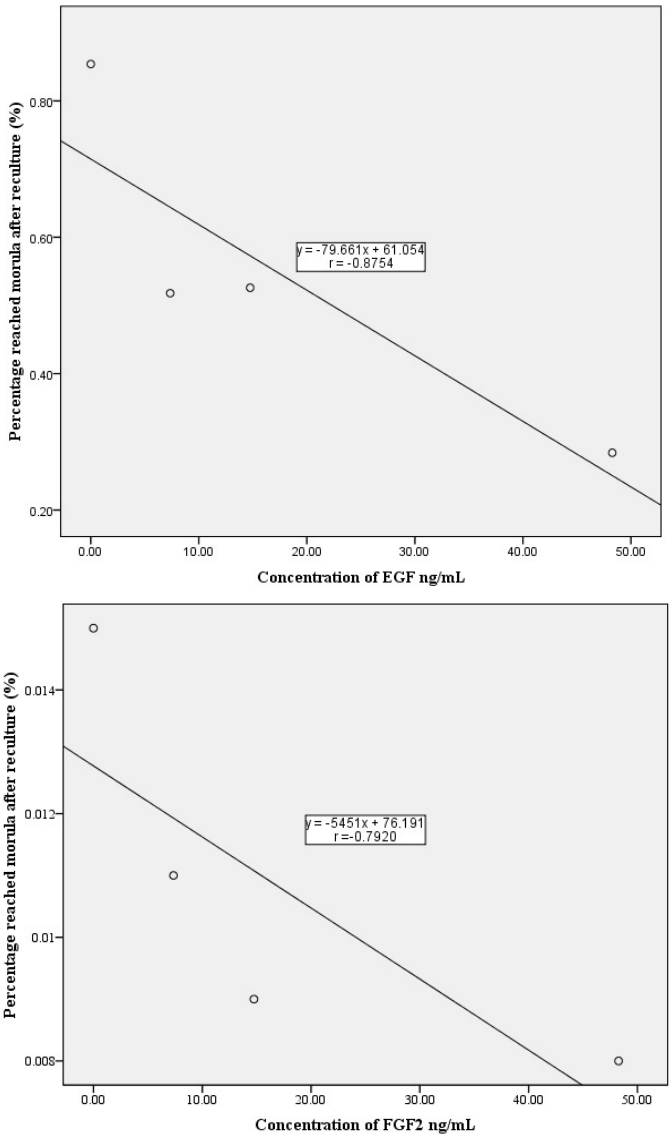

Figure 3: Correlation between concentration of growth factor and percentage of embryos reached morula after reculture (%).

The results showed that after culture for 48 hours Bali cattle embryos developed into 2-, 4-, 8-, and 16-cell (Table 1). The embryonic development of Bali cattle *in vitro* varies in culture 48 hours post insemination. These differences show that early embryo development is a crucial stage for achieving good results. The early stage of embryonic development indicates that cell division is difficult to be done because it involves processes that are dynamically molecularly arranged and undergo complex structural changes (Marsico et al., 2019). In addition, it is suspected that embryonic development abilities are related to the existing growth factors (Prasad et al., 2018). Research results Charpigny et al. (2021) reports several growth factors have been shown to affect the rate of embryonic development, the proportion of embryos that progress to the blastocyst stage, the number of blastocyst cells, metabolism and apoptosis. After re-culture for 48 hours or 96 hours post insemination (Table 2) Embryos of stage 4 cells, 8 cells,

and 16 cells were able to develop reaching the morula stage respectively 13.16%, 29.03%, and 48.28%. This showed that the embryo's developmental ability to reach the morula stage is higher in embryos capable of reaching stage 8 and 16 cells at 48 hours of culture after insemination. The inability of the embryo to develop further is also likely to be the presence of genome activation in the cattle embryo, as explained by Turathum et al. (2021) that cattle embryos undergo their genome activation starting at stage 8-16 cells so that embryonic development to stage 8 cells depends on the maternal genome carried by oocytes. Embryos that are unable to develop to the next stage are likely influenced by the culture medium used, as explained by Abd El-Aziz et al. (2016) that the efficiency of cultures in each laboratory is different and the culture media is very influential on the final result of the culture round. In addition, one of the factors that support cell division is the quality of oocytes and it can predict the potential development of the embryo produced (Da Broi et al., 2018).

Table 2: The development of embryo bali cattle after re-culture for 48 Hours (96 hpi).

Cell groups	Number of embryos	4 Cells (%)	8 Cells (%)	16 Cells (%)	Morula (%)
2 cells	70	20(28.57)	15(21.43)	5(0.07)	0 (0)
4 cells	68		18(26.47)	15(22.06)	5 (7.35)
8 cells	61			22(36.06)	9(14.75)
16 cells	29				14(48.27)

The concentration of EGF of 0.3 ng/mL found in the follicular fluid proves that EGF is in the follicles of Bali cattle, this result shows that EGF is needed by oocytes in the maturation process, especially in the follicles. Prasad et al. (2018) state that EGF has a positive effect in follicular fluid that can stimulate the meiosis process and during maturation *in vitro*. EGF receptors found in the ovaries and inside the oocyte cumulus complex in pig (Blaha et al., 2015), human (Richani and Gilchrist, 2018) and bovine (Luo et al., 2020). EGF is produced by the endometrium of cattle and expressed by uterine epithelial cells (Arai et al., 2013). EGF has the ability to increase the production of prostaglandin E2 (PGE2) and prostaglandin F2 alpha (PGF2α) in both epithelium and stromal cells that play a role in the regulation of Uterine function (Takatsu et al., 2015).

The concentration of EGF in Bali cattle embryo culture media showed that EGF has a role in the development of Bali cattle embryos that are cultured *in vitro*, although they are not found in oocyte maturation medium. The lower the concentration of EGF At each stage of embryonic development, it has an impact on the low ability of the embryo to develop further. The results of the research of Lonergan et al. (1996) that EGF is found in embryo preimplantation. The addition of EGF 100 ng/mL to culture media significantly increases blastocyst (Zahmatkesh et al., 2013). The same has also been reported by Ahumada et al. (2013) that the addition of EGF to culture media increases the competence of *in vitro* culture development of bovine oocytes and 10 ng/mL supplementation in culture media can increase the percentage of cell division at the age of 48 hours after culture in cattle (Prasad et al., 2018).

High concentrations of FGF2 in follicular fluid indicate that FGF2 is needed for the development and maturation of oocytes while inside the follicle, while in maturation media and culture media the concentration of FGF2 shows a downward trend (Figure 2), the concentration of FGF2 in maturation media is higher suspected to be sourced from internal oocytes and cumulus cells. As have been explained by Ahumada et al. (2013) and Barros et al. (2019) that in cattle, the expression of FGF2 is regulated by oocytes in cumulus cells, FGF2 exerts an effect on the survival of cumulus cells and the quality of the extracellular matrix (ECM) during the maturation of oocytes and *in vitro* allows to support developmental competence as well as physiological role during the late stages of COC differentiation. The presence of FGF2 in Bali cattle follicular fluid shows its role in the maturation of oocytes *in vivo*, as this FGF2 plays a role mainly in the selection of dominant follicles, regulating granulosa cell proliferation, differentiation and steroidogenesis (Chang et al., 2016) Exogenous FGF2 can improve the developmental competence of cattle and pig oocytes during IVM (Yuan et al., 2017). Although the degree of expansion of the cumulus is not affected, nevertheless the quality of the cumulus mass is altered by FGF2, since it becomes more difficult to separate cumulus cells and to remove the expanding cumulus from the oocyte. Known ECM is different during *in vitro* maturation and *in vivo* (Dunning et al., 2012). Research by Reineri et al. (2018) on the evaluation of mRNA expression from bone morphogenetic protein 15 (BMP15), Growth factor and differentiation factor 9 (GDF9), FGF2 and its main receptors, altering growth factor 1 beta receptor (TGFβ-R1), bone morphogenetic protein receptor, IB type (BMPR-IB) and fibroblast growth factor receptor 2 (FGFR2) in bovine follicle cells, found in oocyte cumulus and follicle cells in cattle.

FGF2 concentrations found in culture media, FGF2 concentrations are getting lower as the number of cells cultured increases (Figure 2). This shows that FGF2 is thought to have a role in the development of Bali cattle embryos that are cultured *in vitro*. Cooke et al. (2009) explained that fibroblast growth factors (FGFs) were involved in regulating interferon-tau expression (IFNT) and other important events associated with early conceptual development, in cattle the concentration of FGF2 in bovine blastocyst was about 0.037 ng/mL. FGF2 is expressed by

the luminal epithelium and glands detected in the uterine lumens of cattle and ewes along the estrus of the cycle and the beginning of pregnancy (Michael et al., 2006; Ocón-Grove et al., 2008). FGF2 supplementation during maturation and culture is able to enrich the expansion of cumulus cells, the maturation of oocytes and the degree of embryonic division in buffalo (Kumar et al., 2020).

Growth factor concentrations of EGF and FGF2 have a negative correlation with the embryo's ability to develop to morula after reculture (Figure 3). EGF and FGF2 have a strong relationship to the development of Bali cattle embryo cells. The concentration of EGF and FGF2 in embryo culture media decreases as the stage of embryonic development increases, this suggests that EGF and FGF2 are likely to be used by Bali cattle embryos in the process of development. Sakagami et al. (2012) reported the addition of a combination of IGF-1 and EGF to culture media in cattle produced an additive effect and embryos developed into blastocyst at a relatively high rate. The lower concentration of FGF2 compared to EGF in Bali cattle embryo culture *in vitro* shows that EGF plays more of a role in embryo production in Bali cattle. The research of Sugawara et al. (2010) explained that almost no expression of FGF2 was detected in trophoblast tissue, but showed proliferation activity in cell cultures. FGF2 in rabbits is sufficient for isolation and proliferation of rESC strains derived from fertilized embryos and keeping embryos from being differentiated. Nevertheless, the FGF1 and FGF2 present in the culture have an additive effect in maintaining the stem of the rESC line (Lo et al., 2015). FGF1 and FGF2 play an important role during pre-implantation (early period of gardening) in cattle (Chiumia et al., 2020). The findings of Lim et al. (2018) showed that FGF2 increases at the stage of fetal proliferation of cows on day 14 after pregnancy. The existence of FGF2 in cultural media *in vitro* also contributes to the development of Bali cattle embryos *in vitro*. FGF1 and FGF2 are involved in supporting embryonic development during the spatiotemporal period of early pregnancy in cattle (Chiumia et al., 2020). In addition, FGF2 is also related to progesterone in maintaining the viability of granulosa cells and also inhibits apoptosis of epithelial cells on the surface of the ovaries (Evron et al., 2015). Therefore, EGF and FGF2 are present and contribute to embryonic development *in vitro*. Interestingly EGF regulates several uterine functions including endometrial cell proliferation, cell differentiation and prostaglandin production (PG) in some species, these effects are mediated by EGFR present in target cells (Krishnaswamy et al., 2010). The results of the study of Pascarelli et al. (2021) that one mutation in EGF is sufficient to change the activation of the EGFR signaling pathway at the cellular level.

CONCLUSIONS AND RECOMMENDATIONS

Higher EGF concentrations were found in culture media than follicular fluid and maturation media while FGF2 was found to be higher in follicular fluid than maturation media and Bali cattle embryo culture media. The EGF and FGF2 concentration decreases as the embryonic development stage increases.

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NOVELTY STATEMENT

This is the first to report on the developmental ability of balinese cattle embryo produced *in vitro* based on its cell group as well as reports on epidermal growth factor (EGF) and basic fibroblast growth factor (FGF2) profiles in follicular fluid, maturation media and embryo culture of Bali cattle.

AUTHOR'S CONTRIBUTION

ED conceptualizing research, conducting laboratory experiments and examinations, analyzing data, and writing and revising manuscript manually. HS analyzing data and writing manuscripts. SB analyzing data and writing manuscripts. HH conceptualizing research, conducting script writing experiments. EMK conducting laboratory examination, analysing data and writing manuscripts. All authors read and approved the final manuscript.

Conflict of interest

The authors have declared no conflict of interests.

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