Epidermal Growth Factor as Trigger Mitotic Cleavage in Goat Cumulus Cell

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Abstract. The success of oocyte maturation is strongly influenced by the rate of expansion of cumulus cells. Cumulus cell expansion is inseparable from cell multiplication through mitotic division which is triggered by several factors, one of which is the growth factor, namely Epidermal Growth Factor (EGF). An increase in the number of cells undergoing mitosis can be used as an indicator of the quality of cumulus cell expansion. The treatments in this study were EGF concentrations of 0 ng/ml, 50 ng/ml, 100 ng/ml, and 200 ng/ml. Observation method with BrdU-PI (Bromodeoxyuridine-Propidium iodide) Flowcytometry. The results showed that the cells that experienced the most mitosis were EGF 200 ng/ml treatments with the amount of 25.79%.

1. Introduction
Reproductive technology developments such as reproductive biotechnology, namely artificial insemination, estrus synchronization, super ovulation, in vitro maturation (IVM), in vitro fertilization (IVF), in vitro culture (IVC), embryo transfer (TE), and cloning, are expected to help overcome the problem reproductive efficiency [1]. Rumah Prong Hewan (RPH) byproducts from slaughtering goats is an ovary that is beneficial as a cheap and easy source of oocytes was obtained. In vitro fertilization processes include taking oocytes from ovarian follicles, oocyte maturation, spermatozoa capacitation, in vitro fertilization, and already oocyte culture fertilized to become an embryo [2].

During this time the increase in oocyte maturation is mostly done by using hormone induction, growth factors, and cytokines. As a functional unit, the ovarian follicle [3-4-5] is a micro-environment for oocytes consisting of cumulus and granulosa cells. However, more than 99% of the follicles cannot ovulate and experience atresia at the stage of follicular development. In bovine follicles the most prominent is the death of follicular cells namely granulosa and cumulus cells.

Cumulus cell plays an important role during oocyte growth and maturation [6] which is indicated by the significance between the rate of cumulus cell expansion and the quality of oocyte maturation [7]. The role of cumulus cells is as a mediator of energy transport providers [8], mediating the effect of hormones on the cumulus-oocyte complex [9], micronutrients, and/or carrier molecules for oocyte development (Buccione et al., 1990; [10]. Cumulus cells expansion is strongly influenced by cell proliferation, a decrease in cumulus cell proliferation will affect overall oocyte maturity. Cumulus expansion determines the maturity of oocytes, considering that cumulus contains various active ingredients needed by oocytes during meiosis. Estrogen and progesterone hormones produced by granulosa cell culture play a role in initiating DNA from cumulus cells to proliferate [11].
Addition of growth factors to the media of maturation and culture media increases the proliferation and differentiation of cumulus cells [12]. Epidermal Growth Factor (EGF) induction activates EGF receptors (EGF-R) that are important for mitogenesis and cell differentiation associated with increased cell proliferation. Cell proliferation produces two cells that originate from one cell. This condition requires cell growth which is then followed by cell division. Cell proliferation activity can be determined by calculating the mitotic index. [13]. Mitosis is the process of genome division that has been duplicated by cells into two identical cells produced by cell division. Mitosis is generally followed by cytokinesis which divides the cytoplasm and cell membranes. This process produces two identical daughter cells, which have almost the same distribution of organelles and cell components. Mitosis and cytokinesis are the phases of mitosis (M phase) in the cell cycle, where the initial cell is divided into two sapling cells that have the same genetic makeup as the initial cell.

2. Experimental

2.1 Materials
The materials to be used are goat cumulus cells, TCM-199 medium, Epidermal Growth Factor, Phosphate Buffer Salin, washing media and culture media.

2.2. Cumulus cell collection
Collection of goat ovaries from abattoirs. The ovaries are cleaned from other tissues and then taken to the laboratory by putting them into a 0.89% physiological NaCl solution which is added with 0.006 grams of penicillin-G and 0.1 grams of streptomycin sulphate and maintained at 35°C. Complex oocytes collected from follicles with a size of 2-6 mm were performed using a 21 G needle connected to a 10 ml disposable syringe containing 1 ml of 0% serum washing media. Furthermore cumulus cells were washed three times with washing media each washing media 0%, 5%, and 10% serum.

2.3 Cumulus cell culture for observation of mitosis.
The cumulative cell collection results were transferred to a 35 mm culture dish containing culture media with concentrations of 0 ng / ml, 50 ng / ml, 100 ng / ml, and 200 ng / ml, respectively. Furthermore, incubated in an incubator at 38°C, 5% CO2. Observation of cumulus cell mitosis was carried out using the BrdU-PI immunofluorescent method. After the 20th hour culture, 1 ml of 10 µM BrdU (Sigma, Cat. No. B5002) was given into the culture cells and incubated for one hour. After one hour harvesting cumulus cell with 0.5 ml trypsin-EDTA and incubated for three minutes. Trypsin-EDTA is removed and replaced with 1 ml of 0% serum culture media and incubation for two minutes. Furthermore cumulus cells were transferred into 15 ml polypropilene tubes containing PBS for further staining with immunofluorescents with BrdU-PI.

2.4. Immunofluorescent mitosis cumulus cells.
Culture cells in polypropylene tubes were centrifuged (Sartorius Sigma 3-18K) at 600xg (~ 2328 rpm) for 10 minutes and at room temperature. Furthermore, the supernatant is aspirated, and then added 0.5 ml of 70% chilled ethanol to the pellet and incubated for 20 minutes at room temperature. The pellets are washed with 1 ml washing buffer (PBS-0.5% BSA) and re-centrifuged at 600 x g for 5 minutes. The supernatant is aspirated and the pellet is resuspended in 0.5 ml denaturing solution (2M HCL) and incubated for 20 minutes at room temperature. Add 1 ml washing buffer and re-centrifuged at 600xg for 5 minutes. Pellets are taken and suspended in 0.5 ml 0.1 M sodium borate (N2B4O7) pH 8.5 to neutralize the acidic residual content. Incubate for two minutes at room temperature. Add 1 ml wash buffer homogeneously well and centrifugate at 600xg for 5 minutes.
Added 0.5 ml of purified anti-BrdU monoclonal antibody (Purified anti-BrdU; Biolegend, 335801) in dilution buffer (PBS, 0.05% Tween-20 and 0.05% BSA) with a concentration of 2 µg/100 µl and incubated for 20 minutes at room temperature. Then add 1 ml wash buffer and centrifuge at 600 x g for 5 minutes. Supernatant and pellet aspirations taken plus 0.5 ml dilute antibody secondary FITC-conjugate goat anti-mouse IgG (in dilution buffer with a ratio of 1: 200) then incubate for 20 minutes at room temperature and in a dark room. Next add 1 ml wash buffer and centrifuged at 600xg, for 5 minutes and aspern the supernatant.

The centrifuged pellets were previously resuspended into 0.5 ml of propidium iodide (10 µg/ml in PBS) (Analytical Fluka, 81845) and incubated for 30 minutes at room temperature and protected from light or in a dark room. Cell analysis uses flow cytometry at 488nm and for BrdU-linked green fluorescence (FITC) with 514 nm bandpass filters and DNS linked red fluorescence (PI) at a wavelength of 600 nm. Flow cytometry results in the form of luminescence of cell images detected with BrdU.

3. Results and Discussion

The effect of Epidermal Growth Factor (EGF) as a trigger for mitotic changes in cumulus cells can be seen in Figure 1.

![Figure 1](image_url)

**Figure 1** Cumulus cells mitotic flow cytometry with EGF treatment.
a) EGF 0 ng/ml, B. EGF 50 ng/ml ,. EGF 100 ng/ml, D. EGF 200 ng/ml.

In Fig. 1 Quadrant showing the mitotic division pattern in the lower left quadrant (LL), cells that die in the upper left quadrant (UL), initial apoptosis (UR), and final apoptosis (LR) with the percentage of each can be seen in Table 1. From Table 1. showed that EGF treatment with 100 ng / ml could increase the occurrence of mitotic division in cumulus cells. The cell cycle is a vital process in the life of every organism. Normally, the cell cycle produces cell division. In general, cell division is divided into two stages, Mitosis (M), which is the division of one cell into two cells; and Interphase, the process between two mitoses. Interphase consists of gap1 (G1) phase, DNA synthesis (S), and gap2 (G2) [14].
Table 1. Percentage of mitosis in cumulus cells by EGF treatment

| EGF (ng/ml) | Mitotic of cumulus cells (%) |
|-------------|----------------------------|
| 0           | 2.13 ± 0.11*                |
| 50          | 0.45 ± 0.12*                |
| 100         | 7.48 ± 0.00b                |
| 200         | 25.79 ± 0.00c               |

The cell cycle has two main phases, namely the S phase (synthesis) and the M phase (mitosis). Phase S is the phase of replication of chromosomal DNA in cells, whereas in phase M there is a separation of the two sets of DNA chromosomes into two cells. The phase that limits the two main phases is called the Gap. G1 is present before the S phase and after the S phase is called G2. In the G1 phase, cells make preparations for DNA synthesis which is the initial phase of the cell cycle. The marker of this phase is the expression and synthesis of proteins in preparation for entering S phase. In phase G2, cells carry out further synthesis for the division process in phase M.

![Figure 2. Percentage of dead cumulus cells, early apoptosis, final apoptosis, and mitosis.](image)

Cells that multiply will enter the next cell division or exit into the G0 phase. Cells that actively reproduce have at least two critical points, the first in the G2 phase and the second in G1, while the cells entering the cycle of G0 (resting phase) are the critical points in the G1 phase [15]. From the results of the study showed the most effective EGF to trigger mitotic division at a concentration of 200 ng/ml with a percentage of 25.79%. Fertilization of spermatozoa in cattle oocytes that have complete cumulus cells of 78% causes an incidence of polyspermi by 8% [16]. With the increasing number of cumulus cells that support oocytes in fertilization can reduce the incidence of polyspermi, so an increase in mitotic division is very important in the success of fertilization. This proves that the addition of EGF to culture media can mediate the occurrence of mitosis in cumulus cells in vitro.

Growth factors are ligands in the form of proteins that bind to receptors or enzymes related to receptors on the cell surface that cause various cellular responses such as cell proliferation and differentiation.

In Fig. 2 is the result of EGF treatment of the percentage of phase on proliferation. The higher concentration of EGF shows an increase in the number of mitoses and a decrease in the number of dead cells. The increase in the percentage of mitosis shows an increase in cumulus cell proliferation.
and indicates that the quality of cumulus cell expansion is increasing. This means that the function as a microenvironment for oocyte maturation is increasing. In addition, mistakes during mitosis can cause the production of daughter cells with too many or too few chromosomes, a condition known as aneuploidy [17] and are associated with aging and tumorigenesis [18]. Cells exhibit structural changes that often occur on chromosomes including deletion, amplification, and translocation.

Mistakes in mitosis are the main source of numerical changes in the number of chromosomes observed in cancer and have also been recognized recently as a contributing factor in the generation of back chromosomes rangelings [19][20]. The main function of cumulus cells is to provide nutrients to mature oocytes. Sometimes tumors can form from cumulus cells. Cumulus cell tumors are smooth and round in appearance. Internally, the tumor contains cysts that can fill with blood. Tumors cause changes in estrogen levels, which are detected throughout the body.

4. Conclusions
Epidermal Growth Factor treatment on cumulus cells can trigger an increase in mitotic division with a percentage of 25.79% at an EGF concentration of 200 ng/ml.

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