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Utilization of hepatocyte cell culture to increase the success of *in vitro* fertilization in cattle

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ABSTRACT

This study aimed to determine levels of IGF-1 and estrogen obtained from hepatocyte cells culture for bovine *in vitro* fertilization. Hepatocyte cells were cultured in TCM 199 + FCS 10% + BSA 10% to produce IGF-1, estrogen and progesterone. Progesterone concentration was minimized by binding technique using antiprogesterone coated polystyrene tubes. The IGF-1 and estrogen are used as supplementation media for *in vitro* fertilization and as embryo culture media. The result showed that 6 days incubation period had the most confluent monolayer compared to the other incubation period, therefore, resulted in the highest concentration of IGF-1 as well as estrogen. AntiP4 could be used to absorb progesterone produced from monolayer culture of liver and cumulus cells. IGF-1 and estrogen from monolayer culture of hepatocyte cells before and after progesterone absorption resulted in a cleavage rate of 27.48% and 53.61%, respectively. The stage morula embryo rates were 5.73% and 27.59% and could also reduce the apoptosis in the embryos cultured in liver cell were 23.71%, while without supplemented apoptotic rate in embryo cell was 70.58%. It could be concluded that monolayer culture of bovine hepatocyte cells could produce IGF-1 and estrogen growth factor which acted as mitogenic substances that could be used as supplement media in *in vitro* fertilization and embryo development as well as able to reduce the incidence of apoptosis in embryo resulted.

Introduction

Ovaries, which are waste from slaughterhouses, can be used as reproductive organs through *in vitro* fertilization in veterinary medicine to produce more food from beef cattle (Nourin *et al*., 2024). Furthermore, liver waste can be utilized to create growth factors like insulin-like growth factor-1 (IGF-1) and natural steroid hormones like estrogen and progesterone through monolayer cultures of hepatocyte cells using specific media thanks to advancements in biotechnology, particularly molecular biology (Adamek and Kasprzak, 2018).

IGF-1 is a blood-circulating endocrine hormone that is mostly produced in the liver (Ohlsson *et al*., 2009). A rise in growth hormone (GH) will cause the liver to secrete more IGF-1 (Ma and Stanley, 2023). GH controls the release of IGF-1, which in turn controls the formation of bones and extraskeletal tissue (Locatelli and Bianchi, 2014). IGF-1 functions as a growth factor that regulates postnatal growth by promoting skeletal growth via chondrocyte proliferation and extraskeletal tissue growth via protein synthesis and cell division (Racine and Serrat, 2020). Several tissues that function in an endocrine, paracrine, and autocrine way, including cells in the ovaries, Fallopian tube epithelial cells, and endometrial epithelial cells, in addition to liver cells, create IGF-1 (Afradiasbagharani *et al*., 2022). Monolayer cultures of cow cumulus cells have been used in research to produce IGF-1 and steroid hormones; however, monolayer cultures of cow liver cells have never been used to produce IGF-1 and the sex steroid hormones progesterone and estrogen.

Although the liver is the body's second largest organ and serves as the primary source of growth hormones like IGF-1, it has only been utilized as an animal protein source up until now (Caputo *et al*., 2021). Research must thus be done to use the liver, which plays a more significant role as the primary generator of IGF-1, by cultivating liver cells (hepatocytes) in a monolayer culture to create both IGF-1 and the sex steroid hormones progesterone and estrogen. Furthermore, the liver is used in research as a substitute for the ovaries, which are also organs that produce IGF-1 and the sex steroid hormones progesterone and estrogen. These organs are waste products from slaughterhouses, but they are hard to get (Ipsa *et al*., 2019).

Monolayer culture of liver cells (hepatocytes) produces IGF-1 and the sex steroid hormone estrogen as a growth factor, both of which are mitogenic and antiapoptotic substances (Bleach *et al*., 2021). According to scientific theory, the liver is the main source of IGF-1 and the location where cholesterol, the main component of the sex steroid hormone estrogen, is metabolized (Rhyu and Yu, 2021). This study aimed to determine the level of IGF-1 and estrogen obtained from hepatocyte cells culture for bovine *in vitro* fertilization. By introducing IGF-1 and estrogen from monolayer cultures of bovine liver cells to the fertilization media and monitoring the presence of cleavage and embryo development to the morula stage, as well as by lowering the incidence of apoptosis in embryos resulting from *in vitro* fertilization, the efficacy of the study's findings was demonstrated.

Materials and methods

Research design

This study was conducted from September 2023 to March 2024. Liver and ovary samples of cattle were taken from the Pegirian slaughterhouse, Surabaya. Monolayer culture and *in vitro* fertilization were carried out at the Bovine *in vitro* fertilization Laboratory, Veterinary Reproduction Division, Faculty of Veterinary Medicine, Airlangga University. Examination of IGF-1, estrogen, and progesterone levels was carried out at the Endocrinology Laboratory, Veterinary Reproduction Division, Faculty of Veterinary Medicine, Airlangga University.

This research is a pure experimental research conducted through five stages of research, namely the preparation and harvesting of liver cell monolayer culture, determination of IGF-1 concentration and levels

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of estrogen and progesterone hormones in liver cell monolayer culture, analysis of the effectiveness of progesterone absorption by separating/absorbing progesterone (P4) with 100 µl of anti-progesterone (anti P4), bioactivity test of growth factors (IGF-1) and estrogen from liver cell monolayer culture, as a supplement to embryo culture media for cleavage and embryo development *in vitro*, bioactivity test of IGF-1 and estrogen hormones from liver cell monolayer culture results against apoptosis in bovine embryos.

Sample

A sample of 100 grams of cow liver was made into a monolayer culture with 6 repetitions. During the study, 6 cow ovaries were taken from 20-30 ovaries. From each ovary collection, the cumulus oocyte complex (COC) from follicle aspiration was divided into 6 parts (as repetitions) in the treatment: without absorption or with absorption; without supplementation or with supplementation (apoptosis examination), each containing 6-10 COC in a drop containing *in vitro* oocyte maturation media.

Preparation of hepatocyte cultures

The liver was removed from the membrane and connective tissue, cut and weighed as much as 1 gram. Then ground in a mortar, added 5 ml of physiological NaCl and 10 µl of 0.25% trypsin, left for 10 minutes, poured into a centrifuge tube, then centrifuged for 10 minutes at 3000 rpm. The supernatant was discarded, washed with 10 ml of TC media and 1 ml of FCS, centrifuged again for 10 minutes at 3000 rpm, the washing was done 2 times, then the supernatant was discarded, made a dilution by adding TCM 199 media with a ratio (pellet: TCM 199 = 1: 1). Stirred until homogeneous, then the pellet was diluted again with a dilution of 10 µl pellet + 990 µl TCM 199, then put into a microtube.

The number of hepatocytes was counted using the Thoma method. The concentration was made to be 1.9 x 10^6 cells/ml of media. Then 100 µl of TCM 199 media was put into a 36 mm petri dish, the diluted pellet was added, 100 µl was taken using an Eppendorf micropipette, then put into the prepared TCM 199 media. Then incubated in a 5% CO_2 incubator at a temperature of 38.5°C. Monolayer cell harvesting was carried out on days 3, 6, 9, and 12.

IGF-1 Test

IGF-1 concentration examination was carried out on the monolayer cell culture products of liver cells after the monolayer culture was incubated for 3, 6, 9, and 12 days using the IRMA (Immuno Radio Metric Assay) method (Meinhardt *et al*., 2003). Before the assay was carried out, a washing solution was prepared, namely: 60 ml of washing solution available in the ACTIVE* DSL-2800 Kit was taken, placed in a beaker glass, then 1500 ml of deionized water (dH2O) was added and stored at room temperature (±25°C). The standard and control reagents were each diluted by adding 1 ml of dH_2O.

Assay procedure

Tubes were prepared and labeled for Total Count, tubes coated with anti-IGF-1 were numbered and arranged on a tube rack, each duplicated. Each 50 µl of standard solution, control and sample to be examined was inserted into the appropriate tube. 200 µl of Anti-IGF-1 reagent (125I) was added to all tubes. Then stirred slowly by shaking the tube rack above the vortexer at a speed of 1800 rpm which was carried out at room temperature (±25°C) for 2 hours. The liquid in all tubes was discarded in a radioactive waste container except the Total Count tube, then place the tube on a paper towel upside down for 1-2 minutes until the tube was completely dry. Washing was carried out by adding washing solution to all tubes except the Total Count tube and discarding it back into the radioactive waste container. Washing was repeated until 3 washes were carried out. Then counting was carried out by inserting all tubes alternately into the Gamma counter each for 1 minute.

Calculation of assay results

The results obtained from the Gamma counter are Counts Per Minute (CPM), then the Net CPM and % B/T are calculated using the formula:
Net CPM = Average CPM – Average CPM Standard A (0 ng/ml)
Note: CPM = Count Per Minute
Next, a standard curve is made on logit-log paper where the % B/T value of the standard is the Y axis and the IGF-1 concentration value is the X axis. The IGF-1 concentration of the sample is obtained by placing the % B/T value on the standard curve then drawing a straight line intersecting the X axis to obtain a number that is the IGF-1 concentration of the sample.
% B/T = (Net CPM)/(Average Total CPM)

Estrogen level check

Examination of estrogen hormone levels was carried out on days 3, 6, 9, and 12 using the solid phase Radioimmunoassay (RIA) technique (Newman *et al*., 2019). The sample used was fluid from the liver cell monolayer culture medium.

Progesterone level check

Progesterone hormone levels were examined on days 3, 6, 9, and 12 using the solid-phase RIA technique (Newman *et al*., 2019). The sample used was fluid from the liver cell monolayer culture medium.

Radioimmunoassay procedure

The research product samples and Estrogen Kit / Progesterone Kit were adapted to room temperature. Prepared Total Count (TC) tubes and Non Specific Binding (NSB) tubes without antibody coating. Prepare polypropylene tubes that have been coated with antibodies and numbered (A to G) each with duplication for the calibrator and sample to be examined. Then 100 µl of calibrator A was inserted into the NSB tube and tube A, and in tubes B to G, insert 100 µl of calibrator B to G each according to the tube number. Insert 100 µl of each sample into the prepared tubes. Next, 1 ml of tracer 125I Progesterone or 125I Estradiol 17 β was added to each tube, both the calibrator tube and the sample tube, then shaken with an electric shaker (vortexer) for 5-10 minutes. Then all tubes were left at room temperature for 3 hours. After 3 hours, all the liquid in the assay tube is discarded into the radioactive waste bin by inverting the surface of the tube. To clean the remaining liquid, the tube is left upside down on the blotting paper for approximately 5 minutes. Then each tube is inserted into the Gamma counter to obtain the CPM (count per minute) number, to calculate the hormone levels of the samples being examined.

Calculation of assay results

The calculation of assay results was carried out by making a standard curve on logit-log paper based on the points resulting from the calculation of Net Counts and the percentage of binding from calibrator tubes A to G.
Net Counts = Average CPM – Average CPM NSB
% bond = (Net counts)/(Net MB Counts) x 100%
Keterangan: CPM: Counts Per Minute; MB : Maximum Binding (Calibrator A); SB: Non Spesific Binding
Next, the results of the Gamma counter sample examined in the form of CPM are changed into % binding, then interpolated on the standard curve on logit-log paper, the % binding value is the Y axis and the estro-

gen and progesterone levels are the X axis.

In vitro fertilization

In vitro fertilization using Earle's Balance Salt Solution (EBSS) medium. The matured oocytes were then transferred into EBSS medium in the form of drops/rossets (5 drops) with a volume of 25 µl/drop which already contained 25 µl of spermatozoa suspension in the drop in the middle of the rosette. Oocytes were inserted into the edge of the drop, each drop containing 4-5 complex oocyte cumulus (COC) or mature oocytes. Then covered with liquid paraffin/mineral oil, then incubated in a 5% CO_2 incubator with 95% air pressure at a temperature of 38.5°C for 24 hours, after which observations were made for fertilization. Then transferred into a medium containing a liquid from the culture of liver cell monolayers+TCM 199+10% FCS, incubated in a 5% CO_2 incubator with 95% air pressure at a temperature of 38.5°C. After 48 hours (2 days), observations were made regarding the occurrence of embryo cleavage.

Observation of embryo development was carried out by transferring *in vitro* fertilized cow embryos from the 2-8 cell phase into a petri dish containing a liquid from liver cell culture + TCM 199 + 10% FCS. The embryo culture was incubated in a 5% CO_2 incubator with 95% air pressure at a temperature of 38.5°C. Observation of embryo development was carried out every day and media replacement was carried out every 3 days. The final observation of the embryo was carried out after the embryo reached the morula phase or 6 days after fertilization.

Apoptosis examination with Tunel assay

Testing the bioactivity of monolayer hepatocyte cell cultures against apoptosis in *in vitro* fertilized bovine embryos using the TUNEL Assay using the Apoptag Detection Kit (Sugimura *et al*., 2017).

Embryos from *in vitro* fertilization (IVF) stage 2 cells - morula are taken with a micro pipette and placed on a Poly-L-Lysine coating glass object (micro slides), then covered with a glass cover (both sides are given adhesive with liquid paraffin), then fixed with 4% paraformaldehyde or 10% formalin solution. Then left for several days at a temperature of 4°C until the embryo is completely attached to the glass object (by examining the slide under a microscope). If the embryo has not attached properly, the slide is stored at a temperature of 37°C so that it really sticks and dries. After the embryo has really attached to the glass object, then washed with PBS solution 1X by flowing and sucking the PBS solution around the glass cover using tissue or filter paper. Drop with Pronase for 30 minutes and store at a temperature of 37°C. Wash with PBS 3X. Then drop with Proteinase K 200 µg / ml for 30 minutes at room temperature. Wash with PBS 3X. Add 3% H_2O_2 PBS for 15 minutes at room temperature. Wash with PBS 1X. Add DNA Labeling solution 50 µl for 1-1.5 hours at 37°C. Wash with PBS 1X. Cover with Blocking buffer (BSA) 100 µl for 10 minutes at room temperature. Add Antibody solution 100 µl in a dark room, cover the slide with aluminum foil and store for 1-1.5 hours at room temperature. Wash with PBS 1X. Then add Blocking buffer (BSA) 100 µl. Add Conjugate solution for 30 minutes at room temperature. Wash with PBS 1X. Wash with dH_2O. Add DAB solution for 15 minutes at room temperature. Wash with PBS 1X. Wash with dH_2O. Dry the slide, then examine under an inverted microscope. For counterstain, drop 100 µl of Methyl Green Counterstain solution and store for 3 minutes at room temperature. Examine under an inverted microscope.

Absorption

Absorption was carried out using the method of binding/absorbing progesterone (P4) with 100 µl anti-P4 in a coated polyesterene tube with buffer carbonate with two bindings (2x3 hours).

Progesterone absorption procedure

First, a polyesterene coated tube with buffer carbonate of 1 ml pH 9.2 was prepared (to help the bonding/absorption of antiprogesterone on the inner wall of the polyesterene tube). Then 100 µl of antiprogesterone was added, shaken with a vortexer for 1 minute. Then left for 24 hours at room temperature or 3 hours in a 5% CO_2 incubator at 38.5°C. The remaining liquid was removed by washing with 2 ml of deionized water. Then dried by inverting the tube on tissue paper, then closed so that no moisture (dew) enters, (if not used immediately, it can be stored in the refrigerator and can last up to 2-3 months). A 100 µl liquid sample (monolayer culture result) was inserted into the tube, incubated in a 5% CO_2 incubator at 38.5°C to provide an opportunity for the bonding between progesterone and antiprogesterone, repeated once more so that the incubation was carried out 2x3 hours. Then the percentage (%) of binding is measured. The higher the % binding, the higher/more progesterone is bound by antiprogesterone.

Data Analysis

Before statistical analysis was conducted, normality test was conducted using Kolmogorov-Smirnov one sample test and homogeneity test using Levene's test on the collected data. Data analysis for the first, second, and fourth stages of the study used Univariate Analysis of Variance. For the third stage of research data was analyzed using the unpaired t-test (Independent Samples t Test). For the fifth stage of research data was analyzed using One Way Anova. To determine the significance, it was continued with the 5% High Significant Difference (HSD) Test. All statistical calculations were performed using the SPSS 14.0 for Windows program.

Results

Descriptively, the 6th day culture produced the most hepatocyte cells although not significantly different ($p>0.05$), with IGF-1 and estrogen levels that were also higher ($p<0.05$) compared to other culture durations. While progesterone levels in the culture media were not significantly different ($p>0.05$) between culture durations (Table 1).

Table 2 shows that the percentage of embryo development from *in vitro* fertilization on day 2 (stage 2-8 cells) and day 6 (morula stage) with supplementation of hepatocyte cell culture results that have been absorbed by progesterone hormone is higher ($p<0.05$) compared to without progesterone absorption (Table 2, Figure 1). The percentage of embryo apoptosis with supplementation of hepatocyte cell culture results that have been absorbed by progesterone hormone is lower ($p<0.05$) compared to without progesterone absorption (Table 3, Figure 2).

Table 1. Percentage of hepatocyte cells in monolayer culture, levels of IGF-1, estrogen and progesterone on several days of culture.

Culture duration	Number of Cells (%)	IGF-1 (ng/ml)	Estrogen (pg/ml)	Progesterone (ng/ml)
3 days	60.00±16.73	76.38±20.54^{b}	69.38±31.64^{ab}	1.00±0.98^{a}
6 days	71.25±10.87	223.44±68.23^{e}	80.94±25.06^{bc}	0.90±0.21^{a}
9 days	62.50±8.56	122.81±75.38^{cd}	51.31±25.63^{ab}	1.00±0.53^{a}
12 days	40.00±0.000	13.31±11.94^{a}	40.13±23.50^{a}	0.64±0.38^{a}

Note: $^{a.b}$Different superscripts in the same column indicate significant differences ($p<0.05$)

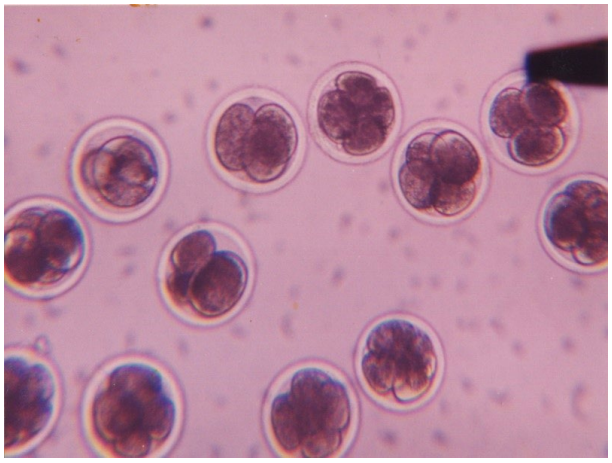

Fig 1. *In vitro* fertilization results, a = 2 cells, b = 4 cells and c = 8 cells. Examination under an inverted microscope Examination under an inverted microscope (Olympus Corporation Tokyo Japan) at 100 x magnification.

Table 2. Percentage of embryo development resulting from *in vitro* fertilization with supplementation of hepatocyte cell culture results without and with absorption of progesterone hormone

	Day 2 (2-8 Cells)	Day 6 (Morula)
Without absorption	26.907±5.995b[a]	5.619±1.876[a]
With absorption	54.833±20.203[b]	28.667±9.416[b]

Note: [a.b]Different superscripts in the same column indicate significant differences (p<0.05)

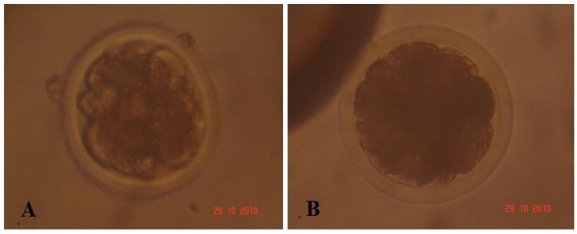

Fig 2. Bovine embryos resulting from *in vitro* fertilization, A: normal 16-cell stage embryo, B: morula embryo undergoing apoptosis (blackish brown color with Tunel assay) (B). Examination using an inverted microscope (Olympus Corporation Tokyo Japan) at 200 x.

Table 3. Percentage of apoptosis of embryos resulting from *in vitro* fertilization with hepatocyte cell culture supplementation without and with absorption of progesterone hormone.

Group	Apoptosis incidence (%)
P0 (Control)	70.575±10.767[b]
P1 (Liver cells)	23.717±5.623[a]

Note: [a.b]Different superscripts in the same column indicate significant differences (p<0.05)

Discussion

In Table 1, the 3-day culture period still has few cells attached, growing and developing on the bottom of the petri dish where the culture is held, so that the number of monolayer cells produced is still low. The 6-day incubation period produces the largest number of cells compared to other culture periods. The 6-day culture period is the optimal time for cells to cover the entire surface of the bottom of the petri dish where the cells are attached. This is what causes the 9- and 12-day culture periods, cells no longer have a place to grow on the bottom of the petri dish so that if the entire surface of the bottom of the petri dish is covered by cells, then the newly grown cells do not have a place to attach, grow and develop, and eventually cell death will occur.

In this study, the presence of IGF-1, estrogen and progesterone can be detected in the monolayer cell culture products of hepatocyte cells. In Table 1, the average IGF-1 levels of hepatocyte cell monolayer cultures at various culture times showed significant differences (p<0.05). The 6-day culture period obtained the highest IGF-1 levels, which were 223.44e±68.233 and were significantly different (p<0.05) compared to the 3, 9, and 12-day culture periods. While the lowest IGF-1 levels (13.31a±11.943) were produced by the 12-day culture period. Of all the culture periods, the IGF-1 levels produced each showed significant differences (p<0.05).

The highest Estrogen hormone levels were obtained from the results of a 6-day culture period (80.94bc±25.059) and the lowest were obtained from a 12-day culture period (40.13a±23.503). The results of the Estrogen hormone from a 6-day culture period were significantly different from the culture periods of 3, 9, and 12 days (p<0.05). While between the culture periods of 3 and 9 days did not show any significant difference (p>0.05). The lowest Progesterone hormone levels were obtained from the results of a 12-day culture period (0.900a±0.209) and did not differ from the results of a 6-day culture period (0.644a±0.379) (p>0.05). Of all the culture periods of 3, 6, 9, and 12 days, each showed no significant difference (p>0.05).

In this study, the presence of IGF-1, estrogen and progesterone can be detected in the monolayer culture product of hepatocyte cells because according to the researcher's argument and from several references, the liver is where cholesterol metabolism occurs which is the main ingredient for the biosynthesis of steroid hormones, namely progesterone, testosterone and estrogen (estradiol), so that with the monolayer culture of hepatocyte cells, estrogen and progesterone hormones can also be produced, although the levels produced are lower than IGF-1 because IGF-1 is mainly produced by the liver (Hu *et al*., 2010).

Table 2 shows that the average number of embryos that developed in hepatocyte cell monolayer culture on day 2 (2-8 cells) without absorption with antiprogesterone was 26.907±5.995, while with absorption it was 54.833±20.203. The average number of embryos that developed into the Morula stage (day 6) cultured in hepatocyte cell monolayer culture without absorption was 5.619±1.876, while with absorption it was 28.667±9.416. These results show a very significant difference (p <0.01) in the development of 2-8 cell embryos and Morula stage embryos, the results of monolayer cultures between those without absorption and those absorbed by antiprogesterone. This is because in the monolayer cell culture media that has been absorbed with antiprogesterone, the antimitogenic effect of progesterone has been removed/bound by antiprogesterone, so that the role of IGF-1 and estrogen in the monolayer cell culture fluid can work perfectly as mitogenic materials, thus producing a greater number of embryos that cleavage and develop into morulas (Peluso and Pappalardo, 1998).

In cattle, embryo developmental block usually occurs at the 8-cell stage. This embryo developmental block is related to the quality of the oocyte cytoplasm (Meirelles *et al*., 2004). By using a media supplement made through a monolayer culture of bovine hepatocyte cells, this developmental block can be prevented. This is proven by the addition of growth factors IGF-1 and estrogen made through a monolayer culture of bovine hepatocyte cells as a media supplement in fertilization and *in vitro* embryo development can increase the number of cleavages and embryo development to the Morula stage.

Table 3 shows the percentage of apoptosis events in bovine embryos cultured in media containing growth factors (IGF-1) and estrogen. The highest monolayer culture results were obtained in the control (P0) which was 70.58% which was significantly different (p<0.05) from the liver cell origin (P1) which was 23.72%. This is in accordance with Sato *et al.* (2018) who have reported the results of their research on IGF-1 as a mitogenic and antiapoptotic material in the ability of cell proliferation in oocyte maturation, production and *in vitro* embryo culture in cows. The activi-

ty or mechanism of the growth factor IGF-1 and estrogen as mitogenic agents works by inhibiting the release of the proapoptotic p53 protein (Kasprzak, 2021). Inhibition of the p53 protein results in inhibition of the p21 transcription factor, which results in the activation of CDK (cyclic dependent kinase) (Al Bitar and Gali-Muhtasib, 2019). Furthermore, activation of CDK causes the cell cycle to occur, resulting in mitosis and cell proliferation and differentiation. This causes cleavage and cell development in embryos resulting from *in vitro* fertilization using media supplements containing growth factors IGF-1 and estrogen made through monolayer culture of bovine liver cells to increase compared to those without using media supplements, and is very significantly different.

Conclusion

Liver cell monolayer culture produces IGF-1, estrogen and progesterone hormones. Antiprogesterone can be used to absorb progesterone (antimitogenic) from IGF-1 and estrogen (mitogenic) from liver cell monolayer culture, so that IGF-1 and estrogen from liver cell monolayer culture can increase the embryo cleavage rate. Growth factor (IGF-1) and estrogen from liver cell monolayer culture as a supplement to fertilization media and *in vitro* embryo development can reduce the incidence of apoptosis in *in vitro* fertilized embryos.

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Conflict of interest

The authors have no conflict of interest to declare.

References

Adamek, A., Kasprzak, A., 2018. Insulin-Like Growth Factor (IGF) System in Liver Diseases. Int. J. Mol. Sci. 19, 1308. doi: 10.3390/ijms19051308.

Afradiasbagharani, P., Hosseini, E., Allahveisi, A., Bazrafkan, M., 2022. The insulin-like growth factor and its players: their functions, significance, and consequences in all aspects of ovarian physiology. Middle East Fertil. Soc. J. 27, 27. doi: 10.1186/s43043-022-00119-1.

Al Bitar, S., Gali-Muhtasib, H., 2019. The Role of the Cyclin Dependent Kinase Inhibitor p21cip1/waf1 in Targeting Cancer: Molecular Mechanisms and Novel Therapeutics. Cancers (Basel) 11, 1475. doi: 10.3390/cancers11101475.

Bleach, R., Sherlock, M., O'Reilly, M.W., McIlroy, M., 2021. Growth Hormone/Insulin Growth Factor Axis in Sex Steroid Associated Disorders and Related Cancers. Front. Cell. Dev. Biol. 9, 630503. doi: 10.3389/fcell.2021.630503.

Caputo, M., Pigni, S., Agosti, E., Daffara, T., Ferrero, A., Filigheddu, N., Prodam, F., 2021. Regulation of GH and GH Signaling by Nutrients. Cells 10, 1376. doi: 10.3390/cells10061376.

Hu, J., Zhang, Z., Shen, W.J., Azhar, S., 2010. Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. Nutr. Metab. (Lond). 7, 47. doi: 10.1186/1743-7075-7-47.

Ipsa, E., Cruzat, V.F., Kagize, J.N., Yovich, J.L., Keane, K.N., 2019. Growth Hormone and Insulin-Like Growth Factor Action in Reproductive Tissues. Front. Endocrinol. (Lausanne) 10, 777. doi: 10.3389/fendo.2019.00777.

Kasprzak, A., 2021. Insulin-Like Growth Factor 1 (IGF-1) Signaling in Glucose Metabolism in Colorectal Cancer. Int. J. Mol. Sci. 22, 6434. doi: 10.3390/ijms22126434.

Locatelli, V., Bianchi, V.E., 2014. Effect of GH/IGF-1 on Bone Metabolism and Osteoporsosis. Int. J. Endocrinol. 2014, 235060. doi: 10.1155/2014/235060.

Ma, I.L., Stanley, T.L., 2023. Growth hormone and nonalcoholic fatty liver disease. Immunometabolism (Cobham) 5, e00030. doi: 10.1097/IN9.0000000000000030.

Meinhardt, U., Eblé, A., Besson, A., Strasburger, C.J., Sraer, J.D., Mullis, P.E., 2003. Regulation of growth-hormone-receptor gene expression by growth hormone and pegvisomant in human mesangial cells. Kidney Int. 64, 421–430. doi: 10.1046/j.1523-1755.2003.00117.x.

Meirelles, F.V., Caetano, A.R., Watanabe, Y.F., Ripamonte, P., Carambula, S.F., Merighe, G.K., Garcia, S.M., 2004. Genome activation and developmental block in bovine embryos. Anim. Reprod. Sci. 82–83, 13–20. doi: 10.1016/j.anireprosci.2004.05.012.

Newman, M., Pratt, S.M., Curran, D.A., Stanczyk, F.Z., 2019. Evaluating urinary estrogen and progesterone metabolites using dried filter paper samples and gas chromatography with tandem mass spectrometry (GC-MS/MS). BMC Chem. 13, 20. doi: 10.1186/s13065-019-0539-1.

Nourin, S., Mohit, Md.M., Zohara, B.F., Islam, Md.F., 2024. Ovum Pick-Up and Ovaries Characterization of Black Bengal Goat from Slaughterhouse. J. Med. Vet. 7, 219–227. doi: 10.20473/jmv.vol7.iss2.2024.219–227.

Ohlsson, C., Mohan, S., Sjögren, K., Tivesten, A., Isgaard, J., Isaksson, O., Jansson, J.O., Svensson, J., 2009. The role of liver-derived insulin-like growth factor-I. Endocr. Rev. 30, 494–535. doi: 10.1210/er.2009-0010.

Peluso, J.J., Pappalardo, A., 1998. Progesterone mediates its anti-mitogenic and anti-apoptotic actions in rat granulosa cells through a progesterone-binding protein with gamma aminobutyric acidA receptor-like features. Biol. Reprod. 58, 1131–1137. doi: 10.1095/biolreprod58.5.1131.

Racine, H.L., Serrat, M.A., 2020. The Actions of IGF-1 in the Growth Plate and Its Role in Postnatal Bone Elongation. Curr. Osteoporos. Rep. 18, 210–227. doi: 10.1007/s11914-020-00570-x.

Rhyu, J., Yu, R., 2021. Newly discovered endocrine functions of the liver. World J. Hepatol. 13, 1611–1628. doi: 10.4254/wjh.v13.i11.1611.

Sato, A., Sarentonglaga, B., Ogata, K., Yamaguchi, M., Hara, A., Atchalalt, K., Sugane, N., Fukumori, R., Nagao, Y., 2018. Effects of insulin-like growth factor-1 on the *in vitro* maturation of canine oocytes. J. Reprod. Dev. 64, 83-88. doi: 10.1262/jrd.2017-145.

Sugimura, S., Akai, T., Imai, K., 2017. Selection of viable *in vitro*-fertilized bovine embryos using time-lapse monitoring in microwell culture dishes. J. Reprod. Dev. 63, 353–357. doi: 10.1262/jrd.2017-041.