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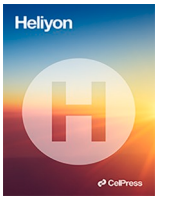

Fibroblast test cells of embryo of Super Java Chicken as an indicator to test toxicity and malignancy

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ABSTRACT

Fibroblast is one component of connective tissue cells in polygonal or stellate with cytoplasmic processes or projections. In vitro, tissue culture is an excellent medium used in biomedical research. The goal of this study was to analyze Toxicity and Malignancy in Embryo of Super Java Chicken as Potential Candidates of Strong Poultry in Indonesia. This study used Preparation of fibroblast primary cell culture of Java super chicken embryos, Pathogenicity test using fibroblast cells from Super Java chicken, Toxicity test using fibroblast cells from Super Java Chicken. Primary fibroblast cell culture of Java super chicken embryos was prepared from 10-day-old brood chicken eggs free of pathogens. Cells were prepared in 96-well microplates with minimal essential medium (MEM) containing 10 % fetal calf serum (FCS) 100 IU/ug penicillin/streptomycinVirus isolates were diluted in stages from 10-2 to 10-5 and inoculated into Java Super Chicken Fibroblast cell culture. The result showed that the negative control of the samples had a faster proliferative power than the fibroblast cell culture of Java super chicken, which was treated with concentrations of 10-2; 10-3; 10-4; 10-5. Moreover, before being inoculated with the virus, the confluent fibroblast cells of Java super chicken looked oval and regular. The day after infection, syncytia (large multinucleated cells) began to form on a small scale and became more pronounced on the second and third post-infection days. CPE was found in the 10-2,10-3 and 10-4 virus dilutions, and CPE was not found in the 10-5 dilution.

1. Introduction

Indonesian people tend to prefer native chicken compared to purebred chicken because of the meat's chewiness, has a high protein content and low fat content [1]. Based on the survey results of the average per capita consumption per week of several important foodstuffs for the period 2007–2014 from the Central Statistics Agency (BPS), the need for consumption of poultry products, especially chicken meat, has increased, especially in the last seven years [2]. Native chicken farms in Indonesia are unable to meet these market demands due to their small business size, limited environmental circumstances, low production, slow expansion, and identifiable nature (incubating and nurturing). The solution that is currently popular is the development of the Java Super chicken, which is the result of crossbreeding between domesticated male chickens and layer chickens [3]. The outcome of this cross is projected to be the elimination of undesirable features, improvement in productivity, and acceleration of the growth of native chickens [4].

In the early 1990s, it was discovered that human cells could be propagated in vitro. Cell culture was inventedin the early '20s as a

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technique for examining animal cells in vitro. Cell culture is related to a complex process of isolating cells from their natural environment (in vivo) and in controlled environmental conditions (in vitro) [5]. Cells from specific tissues or organs can be widely used in research and diagnosis, especially in viral infections [6]. Cell culture is an essentialinstrument in twenty-first-century medicine and diagnosing human infections. Cell culture is a widely used technique in the research of human metabolism and human physiology, which is challenging to do in vivo [7]. Cells can be isolated from tissue then cultured for days to weeks. If clinical procedures and ethical issues permit, cells can be acquired from normal tissue (e.g., skin tissue). Cells can also be obtained from infected tissue (for example, liver tumor cells) taken during surgery as part of therapy for the patient [8].

Cell cultures are usually performed in the form of cell suspensions taken from native tissue (either by enzymatic, mechanical, or chemical dissociation), primary cultures, or cell lines and are carried out in sterile laboratory circumstances with a controlled environment for temperature, gases, and pressure [8]. This must adjust the in vivo environment of the cell so that the cell can survive and proliferate in a controlled manner. Not all research can quickly be done in vivo. Therefore, for research that cannot be done in vivo, it can be done in vitro using cells. Cell culture is instrumental in in vitro research because all studies that use cells must first do cell culture [9].

Fibroblast is one component of connective tissue cells in polygonal or stellate with cytoplasmic processes or projections [10]. Nucleus fibroblasts are oval with one or two active nucleoli to synthesize a glycosaminoglycan base substance matrix. The cytoplasm appears basophilic. The growth of fibroblasts can stimulate cells derived from mesenchyme. In vitro, tissue culture is an excellent medium used in biomedical research. For fibroblast cell culture, the most ideal and easy to do is chicken embryos aged 11–12 days [11]. Fibroblasts in culture media will proliferate and reach confluent after 24 h. This study aimed to analyze Toxicity and Malignancy in Embryo of Super Java Chicken as Potential Candidates of Strong Poultry in Indonesia.

2. Materials and methods

2.1. Preparation of fibroblast primary cell culture of JavaSuper chicken embryos

This study prepared primary fibroblast cell culture of Java super chicken embryos from 10-day-old brood chicken eggs free of pathogens. The reason why this study used primary fibroblast cell because of several reasons. First, chicken embryo fibroblasts (CEFs) are easy to grow and maintain by isolating them from chick embryos and growing in a variety of cell culture media. They are also relatively resistant to infection by viruses, which makes them a good choice for virus research. Moreover, CEFs can be repeatedly passaged, means that they can be used for multiple experiments.

Cells were prepared in 96-well microplates with minimal essential medium (MEM) containing 10 % fetal calf serum (FCS) 100 IU/ug penicillin/streptomycin. After it, the cell is cultured at 37 °C in a CO2 incubator until it reaches confluent, characterized by healthy cell growth and filling the microplate wells.

2.2. Pathogenicity test using fibroblast cells from Super Java Chicken

This study used fibroblast cell culture using cell from Super Java Chicken following the stuyd conducted by Ref. [9]. Fibroblast cells from Super Java Chicken were seeded 16 h prior to infection in triplicate wells of six-well plates at a cell density of approximately 1.7 × 105 cells/well. The cells were infected with NDV strains at MOI of 1 and incubated at 37 °C and 5 % CO2 for 1 h. CPE is observed for 24–72 h.

2.3. Toxicity test using fibroblast cells from Super Java Chicken

Fibroblast cells from Super Java chicken were divided into two treatment groups, namely the control and treatment groups. The viability of each treatment group was calculated using CD50.

2.4. RNA virus inoculation in Java Super Chicken Fibroblast cell culture

The virus used in this study was the Indonesian isolate Newcastle (NDV). Virus isolates were diluted in stages from 10-2 to 10-5 and inoculated into Java Super Chicken Fibroblast cell culture. Cells were incubated using a CO2 incubator for 4 days at 37 °C. Observations were made for 4 days by observing changes in the cells with the formation of Cytopathic effect (CPE).

2.5. Virus harvest and hemagglutination (HA) test

After incubation for 3 days of post-inoculation, the Java Super Chicken Fibroblast cell culture that had been inoculated with RNA virus isolate was harvested usingcell fluid collection with each dilution and continued with HA testing.

2.6. Cell viability and doubling population time

The cultured cell media were discarded and washed with 0.9 % NaCl twice, then 500 μl of trypsin EDTA 0.25 % was added. Then it was shaken slowly and incubated at 37 °C and 5 % CO2 for 3 min. The second stage is centrifugation, which separates cells with components that are not needed. The trypsinated result was placed in a centrifuge tube, and 0.9% NaCl was added. The tube was

centrifuged at 3500 rpm for 10 min. Then the supernatant was removed and the pellet was taken as much as 20 μl to be stained with 20 μl 0.4 % trypan blue and observed using a microscope. Live cells and dead cells (stained) were counted using a hand counter.

3. Results

In this study, fibroblast cell culture was carried out using Java super chicken embryos. Fibroblast cell culture using Java super chicken embryos had different characteristics between treatment groups (Fig. 1). Fig. 1 shows that in 1A, the super chicken fibroblast cells was normal. In 1B, fibroblast cells started to showed condition caused by syncytial effect, while in Fig. 1C fibroblast cells showed the condition caused by Cytopathogenic effects. In Fig. 1D, fibroblast cells showed the condition of*Hem adsorption*.

Toxicity tests were also performed in this study using fibroblast cell culture and Java super chicken embryos. The description of fibroblast cells used as a toxicity test can be seen in Fig. 2. It can be seen from Fig. 2 that there were two conditions of chicken's fibroblast cells, normal Java super chicken fibroblast cells showed by Fig. 2A, and Java super chicken fibroblast cells after toxicity test showed by Fig. 2B.

Table 1 shows that the viability test was carried out on fibroblast cell culture of Java super chicken. In this study, Java super chicken fibroblast cell culture was divided into two treatment groups: control or no treatment and treated Java super chicken fibroblast cell culture. The test results found that in the control group, viability was obtained by 100% and in the fibroblast cell culture group of Super Java chicken, viability was less than 100% (see Table 2).

Able 2 shows that RNA virus inoculation was performed on fibroblast cell culture of Super Java chicken. Virus RNA was diluted to 10-2; 10-3, 10-4 and 10-5. After being diluted, the RNA virus was inoculated in confluent fibroblast cell culture of Java super chicken. This research showed that for the negative control, there was no picture of CPE or cytopathogenic effect. At concentrations of 10-2 to 10-5, a cytopathogenic effect was found.

The HA titer was tested on fibroblast cells of Super Java chicken, showed by Table 3. It was found that the RNA virus with a dilution of 10-2 had a value of 8 HA, 10-3 had a value of 4 HA, a dilution of 10-4 had a value of 2 HA and the highest dilution or 10- had no HA value.

The population doubling time on fibroblast cell culture of super java chicken is also calculated, showed by Table 4. The calculation of Population Doubling time aims to determine the level of proliferation of Java super chicken cell culture before being inoculated with the virus and with some viral retailing in Java super chicken fibroblast cell culture. It showed that super Java chicken fibroblast cell culture has a Population Doubling time value of 1.25; on the 10-2 virus dilution, the Population Doubling time value was 2.5; Virus dilution 10-3 obtained Population Doubling time value of 3.0; 10-4 the Population Doubling time value is 3.0 and 10-5 the Population Doubling time value is 3.0.

4. Discussion

According to the research findings, the CPE picture in the fibroblast cell culture of Super Java chicken (Figs. 1 and 2), before being inoculated with the virus, the confluent fibroblast cells of Java super chicken looked oval and regular. However, at the day after infection, syncytia (large multinucleated cells) began to form on a small scale and became more pronounced on the second and third post-infection days. Meanwhile, another cytopathic effect was in the form of cell detachment and appeared maximal on day 3.

Virus replication in Super Java chicken fibroblast cell culture was characterized by various cell damage, including the cytopathic effect (CPE) and syncytium formation. Infected cells fuse with surrounding normal cells when viral proteins are expressed on their

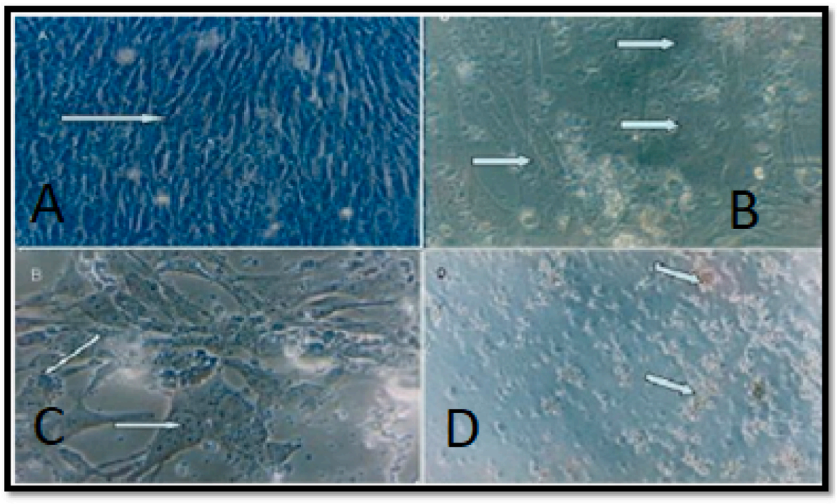

Fig. 1. Java super chicken fibroblast cells after being infected with a virus.

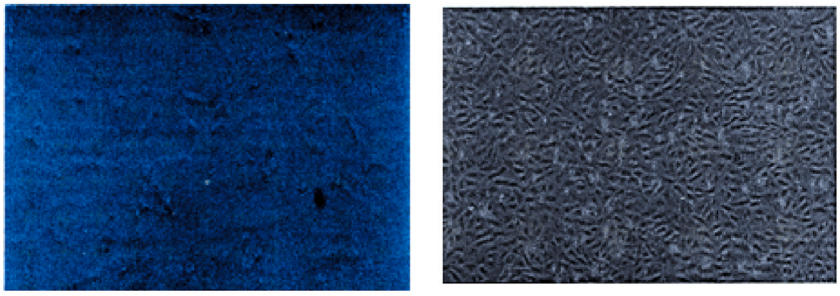

Fig. 2. Java super chicken fibroblast cells were used for toxicity test.

Table 1
Viability of Fibroblast cells from Super Java chicken as a toxicity test.

Treatment group	Number of treatments	Cell Viability (%)
Control	3	100
Treatment	3	93

Table 2
Results of RNA virus inoculation in fibroblast cell culture of Super Java Chicken.

Treatment group	Virus Dilution	CPE or not
Negative Control	–	–
Positive Control	10^{-2}	CPE
Treatment 1	10^{-2}	CPE
Treatment 2	10^{-3}	CPE
Treatment 3	10^{-4}	CPE
Treatment 4	10^{-5}	–

Table 3
Test Results of HA RNA virus titer on fibroblast cells of Super Java Chicken.

Treatment group	Virus Dilution	HA value
Negative Control	–	–
Positive Control	10^{-2}	8 HA
Treatment 1	10^{-2}	8 HA
Treatment 2	10^{-3}	4 HA
Treatment 3	10^{-4}	2 HA
Treatment 4	10^{-5}	0

Table 4
Test Results of Population Doubling time on fibroblast cells of Super Java Chicken.

Treatment group	Virus Dilution	Population Doubling time value
Negative Control	–	1.25
Positive Control	10^{-2}	2.5
Treatment 1	10^{-2}	2.5
Treatment 2	10^{-3}	3.0
Treatment 3	10^{-4}	3.0
Treatment 4	10^{-5}	3.0

surfaces, resulting in large, multinucleated cells known as a syncytium. The syncytium is getting bigger and eventually dies so that it is separated from the wall of the microplate [12]. Fibroblast cell culture as a toxicity test is more sensitive, considered primary cell culture with readily available raw materials and simple culture implementation [13].

One of the main advantages of cell culture is that it can perform physicochemical manipulations (such as temperature, pH, osmotic

pressure, O2, and CO2 levels) and manipulate the physiological environment (such as hormone and nutrient concentrations) in which cells reproduce [14]. The environment during culture is very influential on the growth and development of cells. Another great advantage of cell culture is cell homogeneity and complete control in myogenesis. With increasing approval for the use of cell lines and primary cells, cell culture should be enhanced for in vitro culture in order to contribute more effectively to our understanding of disease biology and therapeutic efficiency [15].

Result above also showed that the control group obtained cell viability of 100%, and the treatment group obtained results of less than 100%. Cell viability is a measure used to determine the ability of cells to survive in a population. Live cells are marked in clear color, and dead cells are blue. It is because the dead cells lose the ability to maintain the integrity and permeability of the plasma membrane. Some of the factors that cause damage to cell permeability are generally influenced by pH, ionic activity in the cells, and the freezing and thawing processes of cells when they are cultured before being treated. Culture media is very important in maintaining cell viability [16].

Virus inoculation was implemented on the fibroblast cell culture of Super Java Chicken to identify whether or not CPE occurred in Java super chicken cell culture. Viruses were diluted to concentrations of 10-2, 10-3, 10-4, and 10-5. The study results found that CPE was found in the 10-2,10-3 and 10-4 virus dilutions, and CPE was not found in the 10-5 dilution. Cell culture is inoculated with cytopathic virus, and then the viral infection spreads through the media to infect starting from adjacent cells until all cells can be infected. The result of cell damage is a cytopathic effect (CPE). CPE can be observed directly with a microscope. This CPE formation is visible from day 1 of post-inoculation. Furthermore, on the second-day postinoculation, both the cells started to break down due to the presence of CPE, and the cells continued to peel.

HA testing was also carried out on viral supernatants inoculated on fibroblast cell culture. The HA value was obtained in the 10-2, 10-3, and 10-4 dilutions, and the 10-5 did not get the HA value. The HA value is a method for determining the virus titer. After being incubated for 3 days, the cell fluid was harvested and collected based on its dilution and continued with HA testing; the cells in the microplate were frozen 3 times, then the tissue culture fluid could be harvested and continued with HA titer testing. Cell fluid is harvested by collecting the fluid according to the level of dilution at the time of inoculation. The cell fluid is collected on a sterile Eppendorf. To then proceed to test the HA titer. When the HA test was carried out, it turned out that only the 10-2, 10-3, 10-4 dilutions both showed a virus titer of 0. It was due to too little virus content during the dilution process of the isolate used for inoculation [17].

Population doubling time was measured on fibroblast cell culture of Super Java chicken. The research results showed thatthe negative control or without the virus had a faster proliferative power than the fibroblast cell culture of Java super chicken, which was treated with concentrations of 10-2; 10-3; 10-4; 10-5. The population doubling time value represents the amount of time required for the cell population to double its original number. If the population doubling time value is high, the proliferation rate is low; if it is low, the proliferation rate is high. Cell viability and population doubling time are often used as parameters to evaluate the success of a cell culture and cytotoxicity markers of a material [18] [19]. This cytotoxicity test is useful to determine whether the material's biological properties are toxic to certain cells or not. One of the indications of cytotoxicity of a material is a decrease in cell proliferation and a decrease in viability [20].

5. Conclusion

According to the findings, Java super chicken, particularly fibroblast cell culture, can be used as a Toxicity and Malignancy test on livestock in Indonesia. The negative control or without the virus had a faster proliferative power than the fibroblast cell culture of Java super chicken, which was treated with concentrations of 10-2; 10-3; 10-4; 10-5. Moreover, findings above showed that before being inoculated with the virus, the confluent fibroblast cells of Java super chicken looked oval and regular. The day after infection, syncytia (large multinucleated cells) began to form on a small scale and became more pronounced on the second and third post-infection days. CPE was found in the 10-2,10-3 and 10-4 virus dilutions, and CPE was not found in the 10-5 dilution. It is based on research that included the development of Java super chicken fibroblast cell culture, inoculation tests, HA tests, viability tests, and population doubling time.

Declaration of ethical clearance

This study used the data bank of cultured cells from Institute of Tropical disease of Airlangga University that has been accredited in following ethical principle of using animal cell as sample of research. This study has not directly conducted the cultured cells by slaughtering chicken. Instead, it was gathering the cultured cell bank from Institute of Tropical Disease. Link to the accreditation certificate of ethic could be found in link below:

https://itd.unair.ac.id/itd/id/

CRediT authorship contribution statement

Maslichah Mafruchati: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Visualization, Writing – original draft. **Nor Hayati Othman:** Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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