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Use of Collagenase Enzyme as a Substitute for Trypsin on In Vitro Growth of Liver, Kidney, and Fibroblasts of Chicken Embryo Cells

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ABSTRACT

This study aims to determine the effect of using the collagenase enzyme on the viability of chicken embryos' liver, kidney, and fibroblast cells in vitro. Liver and kidney organs were collected from embryonic chicken eggs at 15 and 19 days. Meanwhile, fibroblast cells were obtained using muscle tissue of 11-day-old chicken embryos. Each organ and tissue was chopped and incubated using 0.1% trypsin for 10 minutes and 0.1% collagenase for 30 minutes. Concentration, viability, and proliferation rate were calculated after incubation and analyzed using a t-test. The results showed that there was a significant increase in the tissue incubated with collagenase on the viability and proliferation rate of liver cells, kidney cells, and chicken embryo fibroblast cells ($t<0.05$), and not significant in the acquisition of cell concentrations ($t>0$, 05). Cell isolation and purification techniques need to be carried out to study the quality of somatic cell culture of chicken embryos more specifically.

Keywords: collagenase, trypsin, chicken embryos, viability, cell culture.

INTRODUCTION

Since 350 BC, the domestic chicken (*Gallus-gallus domesticus*) has been used as an experimental animal model for research purposes. Its fast-growing embryos compared to mammals make it a strong model organism in various research subjects, such as in understanding cell development and interactions (Weeke-Klimp et al., 2010), genomes (Cogburn et al., 2003), medicine (Ribatti, 2012), immunology, behavior, and reproduction (Burt and Pourquie, 2003; Li et al., 2016).

One of the uses of embryonic chicken eggs in its application is to make it a primary cell culture preparation. The liver and kidney are organs of chicken embryos that are often used as preparations for cell culture and are used in various studies such as the study of toxic agents (Liao et al., 2020), isolating and studying viruses (Soumyalekshmi et al., 2014; Chen et al., 2019) and vaccine development (Xia et al., 2018). Meanwhile, chicken embryo muscle tissue is a source of fibroblast cell culture, which is also often used in studying viruses (Zhao et al., 2019), oxidative stress (Wu et al., 2018), and molecular studies (Chen et al., 2016).

Chicken embryo cell culture has become one of the most important media in preclinical studies, and its application requires a lot of time and accuracy in choosing the right cell type, growth medium, and culture treatment (Srivastava, 2018). The enzymatic reaction is one of the protocols that must be carried out in making cell culture preparations. The enzyme that is commonly used in making primary cell cultures is the trypsin enzyme. However, this enzyme is toxic and can provoke ultrastructural changes on the cell surface, releasing glycoproteins and sugars from the cell membrane and preventing the formation of glycoproteins which impact cell membrane damage which in turn reduces the number of living cells (Pavet et al., 1976). This was also reported by Huang et al. (2010), who said that trypsin had a significant impact on subculture cell apoptosis.

Collagenase enzyme is one of the enzymes commonly used as a substitute for trypsin and has given better results. The use of specific collagenase enzymes has been previously reported in culturing sheep endometrial cells (Ananda, 2019). In addition, several other studies have involved the use of collagenase enzymes to obtain some cells such as mouse Leydig cells (Kaiin et al., 2013), germ cells in horse testes (Jung and Yoon, 2016), and chondrocyte cells (Yonenaga et al., 2017). This is because the collagenase enzyme can make cells aggregate without damaging the cell membrane (Alipour et al., 2016) by breaking the triple helix domain of collagen (Daboor et al., 2010). However, collagenase enzymes in obtaining liver cells, kidney cells, and chicken embryo fibroblast cells have not been widely reported, so further research is needed to determine the effectiveness of using collagenase enzymes in obtaining cells ready for culture.

MATERIAL AND METHOD

Preparation of Liver Cells, Kidney Cells, and Chicken Embryo Fibroblast Cells

Liver and kidney samples were collected from 15-day and 19-day-old chicken eggs, respectively, while muscle tissue samples were collected from 11-day-old embryonated chicken eggs. Each sample was rinsed using Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma, D-8537) containing 62.5 g/ml gentamicin (Sigma, G-1272) and then chopped using scissors and a sterile scalpel. The results were then put into two sterile Erlenmeyer flasks (pumpkin A and flask B), which already contained a magnetic bar and 50 ml of DPBS, then rotated (stirrer) for 1 minute to remove residual blood and fat. The solution was let stand for 1 minute to settle the tissue to the bottom of the flask. Then, the supernatant was removed slowly.

Enzymatic Treatment and Culture

100 ml of trypsin enzyme (Merck, 85450C) was added to flask A and stirred for 10 minutes at room temperature, and 0.1% collagenase enzyme (Gibco, 17018029) was added 100 ml into flask B. Each flask was then rotated (stirrer) for 30 minutes at room temperature and allowed to stand for 1 minute. The supernatant was collected and centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 5 ml of Minimum Essential Medium (MEM) (Gibco, 11095080) containing 4% Fetal Bovine Serum (FBS) (Gibco, 10082139). The concentration and cell viability were calculated on the improved Neubauer hemocytometer counting chamber. All cells obtained were cultured at a concentration of 1×106 cells/ml in a T25 cell culture flask (TPP, 90026) and incubated in an incubator (Thermo Scientific) at 37ºC and 5% CO2. After two days of culture, all cells were harvested to calculate the rate of cell proliferation.

Data Analysis

Data in concentration and viability are presented in percentage ± standard deviation. The data were statistically tested using the t-test at a 95% significance level.

RESULT AND DISCUSSION

The results obtained showed no significant difference in the concentration of liver cells ($t<0.05$) even though in the 0.1% trypsin enzyme treatment, the cell yields tended to be higher compared to collagenase. Meanwhile, incubation using 0.1% trypsin enzyme caused an increase in the concentration in kidney cells and fibroblast cells compared to 0.1% collagenase enzyme ($t<0.05$). Administration of 0.1% collagenase enzyme showed a significant increase in the number of live cells in all cells (Table 1).

Collagenase is a non-cytotoxic hydrolytic enzyme produced from Clostridium histolyicum (Bond and Wart, 1984). Collagenase can degrade collagen (Toyoshima et al., 2001) maximally in the pH range of 6.5-7.8 and breaks down extracellular matrix proteins without damaging cell membranes (Limon et al., 1986). However, pure collagenase isolated from bacteria is known to be inefficient in tissue separation due to the high concentration of non-collagenous proteins and other macro-molecules found in the extracellular matrix of connective tissue and epithelial tissue, requiring combination with other protease enzymes (Worthington Biochemical Corporations).

Collagenase consists of several types that are distinguished based on their functions. Type I collagenase isolates epithelial cells, stromal cells, adrenal cells, lung cells, liver cells, and fat cells; type II isolates heart cells, thyroid cells, salivary gland cells, liver cells, bone cells, cartilage cells; type IV isolates insulin receptor cells (Gibco Life Technologies Corporation). The specific receptor target makes this enzyme the best enzyme in isolating some cells ready for culture.

Table 1. Concentration and viability of liver cells, kidney cells, and chicken embryo fibroblast cells after incubation with 0.1% trypsin enzyme and 0.1% collagenase enzyme in vitro

Cells	Parameter	Tripsin 0.1%	Collagenase 0.1%
Liver cells	Concentration (10^6 /ml)	15,82 ± 2,64[a]	14,51 ± 3,30[a]
	Viability (%)	87,09 ± 4,0[a]	93,82 ± 3,43[b]
Kidney cells	Concentration (10^6 /ml)	17,62 ± 1,30[a]	16,38 ± 0,94[b]
	Viability (%)	88,43 ± 1,74[a]	91,31 ± 2,91[b]
Fibroblast cells	Concentration (10^6 /ml)	18,26 ± 1,08[a]	17,34 ± 0,70[b]
	Viability (%)	84,17 ± 2,50[a]	91,15 ± 3,98[b]

Note: ab Different letters in the same line show significant differences (t<0.05; n=9)

Table 2. Proliferation rates of liver cells, kidney cells, and chicken embryo fibroblast cells after incubation with 0.1% trypsin enzyme and 0.1% collagenase enzyme in vitro

Chicken Embryo	Proliferation Rates (%)	
	Collagenase 0,1%	Trypsin 0,1%
Liver cells	79,18±2,76[a]	74,14±2,53[b]
Kidney cells	82,37±1,5[a]	76,80±1,8[b]
Fibroblast cells	84,56±1,18[a]	81,61±1,32[b]

Note: ab Different letters in the same line show significant differences (t<0.05; n=9)

Cell culture techniques require various materials that support cell proliferation and development. In this study, the enzyme used is collagenase type I, which is specific for isolating epithelial cells, stromal cells, and fibroblast cells, the main tissues in the liver, kidneys, and muscles. The results of this study indicate that the collection process of cells incubated with the collagenase enzyme showed a significant difference in the rate of proliferation compared to cells that were incubated with trypsin during the collection process (Table 2). Type I collagenase is known to have a composition consisting of several other protease enzymes such as caseinase, clostripain, and trypsin (Sigma C-0130). The combination of these various enzymes is thought to affect the process of collagen degradation in cells so that it can take place optimally. The number of dead cells obtained in this study increased significantly after the tissue was incubated using the enzyme trypsin (t<0.05) and was directly proportional to the rate of cell proliferation (t<0.05). The trypsin enzyme is allegedly able to provoke ultrastructural changes on the cell surface, releasing glycoproteins and sugars from the cell membrane and preventing the formation of glycoproteins that impact cell membrane damage (Pevet et al., 1976). Huang et al. (2010) also revealed that trypsin had a significant impact on the amount of subculture cell apoptosis.

CONCLUSION

The use of collagenase enzymes in cell collection and preparation can increase the viability and proliferation of liver cells, kidney cells, and chicken embryo fibroblast cells so that they are effectively used in obtaining cells ready for culture.

CONFLICT OF INTEREST

The authors whose names are listed have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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