The effect of Indonesian honey Tetragonula sp. and Indonesian royal jelly Apis mellifera (Ceiba pentandra) to human preputium cell proliferation in serum-free DMEM

Q S Fachrani¹, M A Dhifanra¹, Y Nugraha², R Yulianti², M Sahlan³, S Pambudi⁴, A Nurhasanah⁴, A Pramono*²

¹Program Studi Sarjanaedokteran, Medicine Faculty, Universitas Pembangunan Nasional Veteran Jakarta, Jl. RS Fatmawati, Pondok Labu, South Jakarta, Indonesia
²Medicine Faculty, Universitas Pembangunan Nasional Veteran Jakarta, Jl. RS Fatmawati, Pondok Labu, South Jakarta, Indonesia
³Department of Chemical Engineering, Faculty of Engineering, Depok, 16424, Universitas Indonesia
⁴Laboratoria Pengembangan Teknologi Industri Agro Dan Biomedika (LABTIAB), Balai Pengkajian dan Penerapan Teknologi Indonesia, Serpong, Indonesia

*andri.pramesti@upnvj.co.id

Abstract. Fetal bovine serum (FBS) is widely used for cell culture media, especially its function as a growth supplement with high growth-promoting factors. An optimal culture medium is needed to increase protein transduction. Unfortunately, FBS reported as media contained protease and contaminated with pathogen microorganisms from an animal host. Fibroblast preputium cell is easy to culture and can be a good model for assessing the medium culture system. In this study, FBS was substituted with honey and royal jelly to find an alternative FBS. This study aimed to determine the effectivity of serum-free DMEM medium with honey from Tetragonula sp. and royal jelly from Apis mellifera (Ceiba pentandra) on the proliferation of fibroblasts preputium cells. The research design used true experimental methods. Samples were taken from healthy people. Fibroblast cells were cultured with various concentrations of honey and royal jelly (0.1%, 1%, 5%). The best result of those various concentrations continued until 9 days with continuous checking in every three days measured with Microtetrazolium (MTT) assay test. Fibroblast cells cultured in Tetragonula sp. honey and royal jelly Apis mellifera (Ceiba pentandra) 0.1% medium had a significant difference, with proliferation higher than 1% (p=0.000) and 5% (p=0.000), but did not exceed proliferation with FBS addition medium. Next, cells in DMEM medium with Tetragonula sp. honey and Apis mellifera royal jelly (Ceiba pentandra) 0.1% on the 3rd, 6th, and 9th (p = 0.000; p = 0.000; p = 0.000) had not similar growth to the standard medium with FBS. However, the growth on the 9th day had a significant difference with the DMEM medium without FBS. High sugar in honey can inhibit fibroblast cell proliferation. The addition of other components as needed to optimize proliferation in honey and royal jelly medium. Isolation of active ingredients in honey and royal jelly can function as an alternative development of an effective and safe substitute for FBS.
1. Introduction

An induced pluripotent stem cell is one of the stem cell technology that is growing rapidly. This technique was first carried out by transforming somatic cells such as fibroblasts in rats into embryonic stem cell-like cells that can induce 4 transcription factors (Oct4, Sox2, Klf4, dan MyC) through retroviral regulation. Preputium is one of the easy collective cell sources for making induced pluripotent stem cells. In Indonesia, it's easy to get these skin because this country has a majority population with Muslim religion which routinely doing circumcision for boys. The preputium is carried out to obtain primary fibroblasts to produce pluripotent stem cells potentially.1

Cytokines and growth factors like Basic Fibroblast Growth Factor (BFGF) were produced by fibroblast cells, which boost cell proliferation and resist cell differentiation. The medium that initiates BFGF and convenient to pluripotent stem cell media is the conditioning medium.2 Dulbecco's Modified Eagle Medium (DMEM) is commonly used for fibroblast cell culture by adding Fetal Bovine Serum (FBS). This serum contains nutrition, several growth factors, and hormones that important for growth in cell culture. However, serum usage can carry viruses and prion that are easily contaminated. Furthermore, this serum contains a complement system and antibody, which encourage the culture cells lysis.3,4 In addition, the serum in human adipose tissue-derived stromal cells (hADSCs) cultures could found a Neu5Gc expression which produced CD46, CD55, and CD59, so the cells become cytotoxic.5 A few honey research originated from Indonesia, especially the research regarding honey as culture media for fibroblast cell culture. One of the honeys reported was used as culture media is Tetragonula sp. Honey. It is easy to get and contains glucose, fructose, and sucrose, which is quite high where the component is a good main medium for cell proliferation.6 But if glucose levels are very high, it will inhibit migration and proliferation of human fibroblast cells in wound healing by suppressing JNK phosphorylation regulation that regulates BFGF.7 Researchers also chose to use royal jelly Apis mellifera (Ceiba pentandra). Protein in royal jelly is Major Royal Jelly Protein (MRJP), increasing cell proliferation and inhibiting cell apoptosis. Therefore, this protein is an effective media for stem cell culture.3,8 Royal jelly also works in human antiaging and antioxidant with decreasing stress oxidative in molecularly. Apis mellifera bee (western honey bee) identified as containing 9 of MRJP protein family also docile and easy to breed.8,9

The usage of media with improper nutrition (such as various complements) and long culture time will affect cell culture results. Morphology transformation is used for identifying the changes that happened. One of the characteristics of morphology transformation in a human cell that clearly can be found is irregular flat-shaped.8 One study revealed that royal jelly from Apis mellifera bees is used to substitute FBS medium as the additional media of DMEM.9 The honey from Tetragonula sp. bees also tested as the replacement of Fetal Bovine Serum (FBS) by showing a little effect in 1% of concentrations.10 Whether the culture time has an impact is still unknown. Here we observed the growth of preputium fibroblast cell proliferation in serum-free DMEM by adding both Tetragonula sp. honey and Apis mellifera royal jelly with different concentrations and different culture times.

2. Literature review

2.1. Fetal bovine serum

Fetal bovine serum (FBS) is a fetal blood clot of diffracted cattle.11 FBS has several components such as bovine serum albumin (BSA), which plays an essential role in maintaining a cell culture, a lot of growth factor as a facility for survival and proliferation cell, gamma globulin as an antibody which can bind cells in culture.4
2.2. Fibroblast preputium

The epidermis and dermis are part of the skin layer. Fibroblasts cells found in connective tissue from the dermis layer, which synthesizes a few components of the extracellular matrix such as elastin, collagen, proteoglycans, and reticular.\cite{12}

*Tetragonula* sp.

*Tetragonula* sp. bees are worker bees with characteristics that include black, blackish-brown abdomen, dark clypeus and tegula, black mesoscutum, and mesoscutellum, malar space separates the mandible from compound eyes. *Tetragonula* sp. is a stingless bee. The isolated honey protein *Tetragonula* sp. has antibacterial activity but still has weak antioxidant activity.\cite{13}

Honey

Honey is a natural product with a sweet flavor, high nutrition and is generally consumed for human health. Honey has many functions, such as antimicrobial, anti-inflammatory, wound healing, and antioxidant. The major composition includes 82.4% carbohydrate, 38.5% fructose, 31% glucose, water, and protein. However, the minor components such as flavonoid, polyphenol, organic acid, glucose oxidase, and catalase enzymes, and phenolic compounds.\cite{14}

*Apis mellifera*

*Apis mellifera* bees are living in colonies. They have a black-yellow abdomen consists of three segments. The queen bee has a reddish-brown color. The male bee has younger-colored and very active. These honeybees are very patient, easy to breed, very high productivity of honey, and very resistant to bacterial attack.\cite{15}

Royal jelly

Royal jelly is a substance secreted by the hypopharyngeal gland and honeybee mandible. That is composed of lots of wet water weight, slight lipids and minerals, several proteins and hydrocarbons, and small amounts of vitamins and polyphenols.\cite{16} About 80% of the royal jelly mass is a major royal jelly protein (MRJP), where royal jelly derived from *Apis mellifera* bees contains nine types of MRJP (49-87 kDa). MRJP1 is the most abundant component and is proven as a key for differentiation factor in honeybee caste. The extracted MRJP causes proliferation for some human cells, for example, hepatocyte, myeloid, and monocyte cells.\cite{8}

Research methods

2.3. Powder preparation

Based on previous research, *Tetragonula* sp. honey alone was formed into a powder, and royal jelly *Apis mellifera* alone formed into a powder.\cite{9,10} Researchers this time, made royal jelly *Apis mellifera* and *Tetragonula* sp. honey were mixed with ratio 10:1 concentration into freeze dryer for one week until it forms a powder.

2.4. Medium preparation

Complete growth media DMEM contained FBS 10%, 1% penicillin-streptomycin, 1% fungizone, or amphotericin B. The treatment medium is made with no FBS inside. The treatment medium was added honey and royal jelly made in the formulation powder (freeze-drying methods) by comparison (honey: royal jelly = 1: 10). The blended honey and royal jelly concentrations were 0.1%, 1 %, and 5% (w/v) in a medium with no F
2.5. Cell culture

2.5.1. Culture concentration

Fibroblast preputium cells were cultured with DMEM medium in 96 well plates with a cell density of 1,000 cells/well. This study has two controls and three treatments, with five repetitions for each control and treatment. DMEM blank without FBS as a negative control, DMEM with 10% FBS as a positive control, DMEM with various addition concentration of royal jelly and honey (0.1%, 1%, 5%) as treatments. The medium was replaced every day for three days. On the 3rd day or 72 hours of culture, fibroblast preputium cells' proliferation is measured with an MTT assay test.

2.5.2. Culture time

This study shows two controls, such as DMEM blank without FBS as a negative control and DMEM with 10% FBS as a positive control. There is also treatment from the best results from last various addition concentration treatments, namely, the 0.1% concentration, with 9 repetitions for each control and treatments until 9th day. The medium was replaced, and the proliferation was checked by MTT assay test every three days, on the 3rd, 6th and 9th day.

2.5.3. MTT assay

This is done in a dark place, and the next well plate is wrapped in aluminum foil and incubated for 4 hours in an incubator (37ºC; CO₂ 5%) until it forms formazan crystals. The absorbance can be read using a microplate spectrophotometer with a wavelength of 560 nm.

2.6. Data analysis

The results of this research data were calculated with a formula. The normality test used Shapiro-Wilk, and the homogeneity test used the Levene Test. Suppose the two tests obtained normal and homogeneous data (p > 0.05). In that case, the data can proceed with the parametric statistical test, namely the One Way Anova test, then analyzed with Post Hoc to see if there are significant differences from each group. Conversely, if in this study the data obtained was not normal or not homogeneous (p < 0.05), then a non-parametric alternative statistical test is performed, namely the Kruskal-Wallis test.

3. Result and discussion

3.1. Percentage of cell proliferation on 3rd day

3.1.1. Various concentration

The percentage of fibroblast preputium cell proliferation is shown after observing fibroblast cell proliferation for three days against various medium concentrations of honey and royal jelly (Figure 1.). The results obtained that percentage fibroblast cells with added honey and royal jelly 0.1% had a higher effect on cell proliferation than the percentage fibroblast cell with added honey and royal jelly 1% and 5%, but not greater than the positive control (DMEM with 10% FBS alone). This condition can be caused because honey and royal jelly alone were not sufficient to boost cells' proliferation. An affluent sugar from the medium can also resist fibroblast cell growth. Besides, because of antioxidant compounds, if it occurs excessively, it can become oxidative stress, thereby inhibiting proliferation. The addition of other substances probably needed as a complement medium. Furthermore, excessive antioxidants can lead to oxidative stress, hence inhibiting cell growth. In this study the morphology cells of fibroblast cells proliferation on the 3rd day are shown in Figure 2.
Figure 1. The percentage of fibroblast proliferation with or without the addition of honey and royal jelly with various concentrations. HRJ: Honey/Royal Jelly, FBS: Fetal Bovine Serum, DMEM: Dulbecco's Modified Eagle Medium.

Figure 2. Fibroblast cell morphology is cultured with different concentrations of honey/royal jelly medium on day 3. (a) Cultured with 10% FBS, cells look longer, compact, and a little bit fat. (b) Cultured with DMEM alone, cells look irregular. (c) Cultured with *Tetragonula* sp. honey and royal jelly *Apis mellifera* 0.1%, cells show more rarely. (d) Cultured with *Tetragonula* sp. honey and royal jelly *Apis mellifera* 1%, cells grow short and rare. (e) Cultured with *Tetragonula* sp. honey and royal jelly *Apis mellifera* 5%, all cells almost entirely dead. All pictures were taken with a magnification of 100.
3.1.2. Duration of culture time

3.1.2.2. percentage of cell proliferation on the 6th day

![Figure 3](image)

**Figure 3.** The percentage of fibroblast proliferation on the 6th day with or without addition honey and royal jelly with various concentrations

![Figure 4](image)

**Figure 4.** Fibroblast cells morphology cultured with addition 0.1% honey/royal jelly medium on day 6. (a) Cultured with 10% FBS, cells look a little fat, denser, and longer. (b) Cultured with DMEM alone, cells look irregular, some are long and short, and cells are still spherical, and there appear to be several dead cells. (c) Cultured with *Tetragonula* sp. honey and royal jelly *Apis mellifera* 0.1%, cells look narrow and regular, some dead cells are found. All pictures were taken with a magnification of 100x.
The study continued to observe the result persistency from the previous result that the addition 0.1% honey/royal jelly showed the best result. The percentage of fibroblast preputium cell proliferation on the 6th day is shown in Figure 3. There were no significant changes between the percentages of cell proliferation on the 6th day than the 3rd day. However, the samples’ morphology in negative controls is getting irregularly wider, and the number of cells is dying from insufficient nutrition from the DMEM medium. This explains how honey and royal jelly alone was not adequate to initiate cell proliferation, along with affluent sugar can also resist cells' growth. Moreover, excessive antioxidants can lead to oxidative stress, so that can inhibit cell growth. In this study, the morphology cells of fibroblast cell proliferation are shown in Figure 4.

3.1.2.3. percentage of cell proliferation on the 9th day

The percentage of fibroblast preputium cell proliferation on the 9th day is shown in Figure 5. This means DMEM medium without adding anything (DMEM blank) cannot sustain fibroblast cells' growth in 9 days. The samples' morphology with the addition of honey and royal jelly 0.1% of the cells are still alive, although some dead cells were found. This can occur due to the effect of MJRP in royal jelly, which protects cells from apoptosis through several intracellular signaling factors. In this study, the morphology cells of fibroblast cell proliferation are shown in Figure 6.

**Figure 5.** The percentage of fibroblast proliferation on the 9th day with or without addition honey and royal jelly with various concentrations.
Figure 6. Fibroblast cells morphology cultured with addition 0.1% honey/royal jelly medium on day 6. (a) Cultured with 10% FBS, cells look solid, longer, and a little bit fat. (b) Cultured with DMEM alone, cells look round and floating, describing dead cells. (c) Cultured with Tetragonula sp. honey and royal jelly Apis mellifera 0.1%, cells look narrow and regular, some dead cells are found. All pictures were taken with a magnification of 100x.

4. Conclusion

Combination of Tetragonula sp. honey and royal jelly Apis mellifera addition in 0.1%, 1%, and 5% showed less effect in fibroblast cell proliferation. The duration of culture time did not change the presentation of proliferation and cell morphology viability significantly. The research of optimizes, potent, and secure medium for Fetal Bovine Serum (FBS) replacement in human fibroblast cell cultures need improvement.

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