Immense addition of royal jelly apis mellifera (ceiba pentandra) insufficient to increase fibroblast preputium proliferation

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Abstract. Culture of preputium skin fibroblasts is a good cell source in the manufacture of induced pluripotent stem cells (iPS). Induced pluripotent stem cells (induced pluripotent stem cell / iPSC) is the latest stem cell technology to get the source of stem cells that are equivalent to their pluripotent properties with embryonic cells, without having to consider ethical problems that arise. Culture systems of preputium skin fibroblast, can use a variety of media. Currently, Dulbeccos modified Eagle medium (DMEM) plus Fetal Bovine Serum (FBS), but if the culture is carried out continuously in FBS, it can increase the immune systems resistance. The addition of royal jelly 5 mg / ml of culture medium was not sufficient to increase the fibroblast preputium viability (p=0.05) so that further research to optimize FBS substitute culture medium is needed. This study was conducted to analyze the comparison of the effectiveness of serum free DMEM media with the addition of Apis Mellifera royal jelly bee from Ceiba pentandra flower for preputium skin fibroblasts cell culture. Royal jelly addition in 1%, 2%, and 5% showed less effect in the proliferation of fibroblast cell, compare with serum addition. The optimum medium for Fetal Bovine Serum (FBS) substitution in skin fibroblast cell cultures must continue to be developed, especially for the transduction protein medium. The results of this study are expected to be used to improve transduction protein medium in iPSC engineering.

1. Introduction
Stem cells or stem cells are collections of cells that have not been diffused, and have the capacity to proliferate and transform into certain cells.1 Based on its source of isolation, stem cells are divided into 3 parts, embryonic stem cells (ESC) isolated from blastocysts, extraembryonic stem cells isolated from umbilical cord, placenta and amniotic fluid and adult stem cells (ASC) isolated from adult tissues such as bone marrow, blood, fat and skin2. The ability of stem cells to proliferate and differentiate into these particular cells is an excellent potential for cell transplantation. In healing wounds on the skin of experimental animals, systemic and topical cell transplants can speed up the process.3

Pluripotent stem cells that are induced (induced pluripotent stem cell / iPSC) are the latest stem cell technology that is growing rapidly in the world. This technology was initiated by the discovery of Shinya Yamanaka et al., 2006, which induced cocktails of 4 transcription factors (Oct4, Sox2, Klf4 and MyC) into somatic fibroblasts of mice cells, to convert them into cells that resemble their characteristics with embryonic stem cells.4,5 This discovery is a new breakthrough to get the source of stem cells that are equivalent to their pluripotent properties with embryonic cells, without having to consider the ethical problems that arise. Pluripotent-induced stem cells can also overcome the problem of rejection of the immune system, because it can be engineered from the patient's own body. In addition to regenerative therapy with stem cells, induced pluripotent stem cells can also be used for disease modelling and new drug findings.6,7,8,9 The engineering of pluripotent-induced stem cells (iPSC) can use cells from the patient's own body that are easily isolated such as skin cells or mucus buccal cells. From previous studies, skin cells can be easily engineered into induced pluripotent stem cells.10
One part of the skin that can be used as a source of stem cells is the preputium skin. Indonesia as a country with a majority of its population embraces Islam is routinely doing circumcision to boys. This circumcision process contains medical waste, which is quite a lot of prepuce, because this prepuce can be used as a source of fibroblast cells. Cell isolation from routine prepuce is done to obtain primary fibroblasts and keratinocytes for research purposes. Fibroblasts are the most common cells found in connective tissue and these cells synthesize several extracellular matrix components such as collagen, reticular and elastin. In addition, it also synthesizes anionic macromolecules namely glycosaminoglycans and proteoglycans as well as multiadesif glycoproteins laminin and fibronectin which can encourage cell adhesion on the substrate. In vitro cultures of fibroblast cells were reported to secrete about 175 types of protein. including cytokines and several growth factors such as basic fibroblast growth factors that can stimulate cell proliferation and inhibit cell differentiation.

Culture systems can use a variety of media. At present, Dulbecco's modified Eagle medium (DMEM) plus Fetal Bovine Serum (FBS) is usually used for human fibroblast cell culture. A study showed that continuous human ESC culture in animal serum and high ascorbate levels produced ectopic expression of CD30 (Chung et al., 2010). Inappropriate miHA peptides can be presented directly to self-MHC class I to CD8 + T cells which destroy graft therapy or through APCs that process and present miHA peptides to T cells, resulting in an alloresponse. Therefore, research is needed which aims to avoid immune rejection, serum-free cell culture medium is prepared to replace FBS. The study of the addition of royal jelly in fibroblast culture showed that the medium (2 mg / ml medium) substitute for FBS could function as anti-microbial although fibroblast proliferation did not show significant differences between royal jelly and FBS culture medium. Further research to see the level of effectiveness of royal jelly on fibroblast culture is needed. Therefore the authors are interested in conducting a comparative study of the effectiveness of serum-free DMEM media by adding Apis Mellifera royal jelly bee from Ceiba petandra flower for preputium skin fibroblasts cell culture. This research is expected to provide more knowledge for the optimization of preputium fibroblast skin culture medium, especially for transduction protein process in iPSC engineering.

2. Literature review

2.1 Fibroblast preputium
Fibroblast cells from Preputium are cells that are easy to isolate and serve as raw materials for iPSC engineering. Fibroblast cells are the most common cells found in connective tissue. This cell synthesizes several extracellular matrix components such as collagen, reticular and elastin. These cell cultures secrete metabolites which can trigger cell proliferation and inhibit differentiation, which is one of them is bFGF (Basic Fibroblast Growth Factor).

2.2 Apis Mellifera
Apis Mellifera bees have three pairs or segments of the back of the yellow abdomen. The apis mellifera queen bee has a reddish-brown color. The younger-colored male bee were very active. It has a wingspan of 0.8-0.95 cm, and length of 0.55-0.71 cm. The 6th abdomen was without 'tomentum'. Mellifera bees are very patient and easy to breed. This honeybee has a very high productivity of honey. Apis Mellifera bees is known to be very diligent in cleaning its nest, so that it looks to remain clean and away from the source of the disease. This bee is very resistant to bacterial attack and is very clever in driving night moth pests.

2.3 Royal jelly
Royal jelly is a substance secreted by the hypopharyngeal gland and worker honey bee mandible. These are yellowish, creamy, and acidic with a slight odor and spicy taste, composed of 60-70% wet water weight, 9-18% of protein, 3-8% lipids, 6-18% hydrocarbons, 0.8-3.0% minerals, and small amounts of polyphenols and vitamins. It plays a very important role in determining honey bee caste because larvae that are fed more royal amounts of royal jelly for a longer period develop into large, fertile, and long-lived queens rather than being smaller, sterile, and short-lived. Royal jelly has been shown to have various functional properties such as antibacterial activity, anti-inflammatory properties, vasodilation and hypotensive activity, disinfectant action, antioxidant effects, anti-hypercholesterolaemic activity, and antitumor properties. As a valuable bee product, royal jelly has been incorporated into traditional human medicine and is widely promoted and commercially available as medicine, health food, and cosmetics in many countries, especially in China and Japan.

2.4. Medium culture
DMEM (Dulbecco’s Modified Eagle Medium) is a basal medium consisting of vitamins, amino acids, salt, glucose, and pH indicators. However, this medium does not contain protein or growth agents. This media requires supplementation to become a complete medium. Generally, this media is supplemented with 5-10% Fetal Bovine Serum (FBS). In addition, DMEM also forms a sodium bicarbonate buffer system (3.7 g / L) and certainly requires artificial carbon dioxide levels to keep the pH in the desired range.22

3. Research Methods
3.1 Decontamination and Sample Preparation
Fresh preputium skin samples were stored in PBS containing 1% penicillin-streptomycin antibiotics and incubated at 4°C for 1 hour. Then the skin sample was soaked in povidone iodine, and soaked in 70% EtOH. Decontaminated samples were transferred to DMEM culture medium supplemented with 10% FBS (Fetal Bovine Serum) and 1% penicillin-streptomycin antibiotics and fungizon antimicotics. Furthermore, the sample was transferred to a petri dish filled with 5 ml PBS, with the dermis side at the top and sides of the epidermis below. Blood clots and fatty tissue attached to the sample are removed, then the skin is turned upside down so that the epidemic side above and the dermis is below. The skin pieces were cut until 0.5-1 cm x 1.5-2 cm size.

3.2 Cell isolation by explant
The dermis tissue pieces are planted in a culture dish with a distance of 5 mm from each other, and a minimal amount of medium is added to keep the tissue pieces from floating but each piece of tissue gets a little medium around it. Culture is stored in an incubator at 37°C and 5% CO2 overnight. The next day two mediums were added from the previous number. The medium is then added gradually until the 3rd day, so that the pieces of dermis tissue that have been firmly attached to the base of the culture plate are submerged by the medium. Observations were made on the appearance of cell growth (outgrowth) around explants. If the growth of cells around the explants has reached a minimum of 50% confluence, passivation is carried out with 0.05% trypsin / EDTA. Cell suspension was transferred to a new culture dish with a cell density of 10,000 / cm2, incubated in an incubator at 37 °C and 5% CO2. The culture medium is replaced every 2-3 days with a new medium.

3.3 Cell Culture
Cells were cultured in culture plates with a cell density of 10,000 / cm2 with or without the addition of royal jelly. DMEM without FBS supplemented with royal jelly (concentration of 1 mg / ml medium; 2 mg / medium and 5 mg / medium) were added as a treatment. The addition of FBS was used as a positive control in this study. The medium was replaced every day and on the third day, proliferation of fibroblast cells was observed with calculations using MTT assay.

3.4 Data analysis
The data used in this study is primary data taken from the circumcision clinic in the form of circumcision waste, namely preputium skin. The results of the research data were calculated with formula for living cells count from absorbance data. The normality test is done using Saphiro-Wilk related to sample number (less than 50). The data are normal if p > 0.05. The homogenity test was done using Levene test (the data is homogen if p value are > 0.05 ). If the data is normal and homogenous, then the next step is the One-Way Anova test. If the p value in this test is < 0.05, there is significant correlation between the independent variable and the dependent variable (H0 rejected). If the p value is > 0.05, there is no significant correlation between the independent variable and the dependent variable (H0 accepted). If the p value in this test is < 0.05, the post-hoc test has to be done in order to see the differences between groups. Kruskall-Wallis test is done if the data is not normal and homogenous.

4. Result and Discussion
Results of the fibroblast cell proliferation studies are shown in Table 1 and Figure 1. Fibroblast cell proliferation was observed over 3 days in response to test media containing varying concentrations of royal jelly. Additionally, the presence of FBS alone had the greater impact on cell proliferation compare to the addition with royal jelly. This can be caused because that the addition of royal jelly alone was not sufficient to imboost the proliferation of fibroblast cell. Other substitution probably needed to be add as a complement.
Figure 1. The percentage of living cells in the addition with and without royal jelly with different concentration

5. Conclusion
Royal jelly addition in 1%, 2%, and 5% showed less effect in the proliferation of fibroblast cell, compare with serum addition. The optimize medium for Fetal Bovine Serum (FBS) substitution in skin fibroblast cell cultures must continue to be developed, especially for the transduction protein medium.

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