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Investigation of royal jelly as a promising substitute to fetal bovine serum in cell culture

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ABSTRACT

Royal Jelly (RJ) was studied for its efficiency as a potential alternative to Fetal Bovine Serum (FBS) in cell culture medium. An all natural cell culture medium was formulated by using tender coconut, Royal Jelly and Honey. This medium was studied in comparison with the commonly used DMEM and FBS medium. The results showed that although there was not any significant difference observed between the cell proliferations in the two different cell culture medium, the RJ medium exhibited a strong anti-microbial atmosphere for the cells to grow and proliferate. This study was aimed at promoting a serum- free cell culture medium for tissue culture application.

Keywords: Serum-free medium, royal jelly and honey.

INTRODUCTION

Fetal bovine serum is commonly used as the cell culture medium. It has been estimated that over half a million FBS is produced each year worldwide which approximates to harvesting more than one million fetuses annually. After slaughter of a cow, the mother's uterus is removed and a needle is inserted between the ribs of the fetus inside the uterus directly puncturing the heart and the blood is vacuumed into a sterile bag. Only fetuses over 3 months are used otherwise the heart is considered too small to puncture. This study aims at avoiding animal abuse; hence a serum free cell culture medium was prepared to substitute FBS. Royal jelly (RJ) is secreted by the hypopharyngeal and mandibular glands of worker honeybees [1]. It is a vital food necessary for the development of the queen honeybee. RJ contains a combination of proteins (12-15%), sugars (10-12%), lipids (3-7%), amino acids, vitamins, and minerals [2]. The queen bees that are exclusively fed RJ have an extended lifespan and well developed sexual organs when compared to the short-lived and infertile worker bees. It has been proven to possess a strong anti-bacterial effect *in vitro*. Royalisin, a protein from RJ acts against Gram-positive bacteriae (*Lactobacilus helveticus, Clostridium, Corynebacterium, Leucnostoc, Stafilococcus, Streptococcus*) [3]. *In vitro* efficiency of both RJ and honey against *Staphylococcus aureus* [4] has been confirmed. RJ has enhanced the synthesis of collagen in skin fibroblasts cells with the help of ascorbic acid-2-O-α-glucoside (AA-2G) [5]. Recently, it has been studied that royalactin (57-kDa protein) present in RJ stimulates the differentiation of honeybee larvae into queens with the help of an epidermal growth factor receptor-mediated signaling pathway which increased the body size and ovary development of the larvae [6]. Tender coconut and Honey have high level of glucose that is essential for human fibroblast cells to grow; hence they were used as the cell growth medium.

MATERIALS AND METHODS

2.1 Delipidization of Human Hair:

Delipidization is the process of removing the lipids from human hair in order to extract the protein. 15 g of human hair is first washed with Sodium Dodecyl Sulfate (SDS) and rinsed several times. The hair is then soaked in a mixture of chloroform and methanol taken in the ratio 2:1. After 24 hrs the oil is separated as shown in Figure 1.

Figure 1: The oil that was extracted by Delipidization of Human Hair

2.2 Extraction of Keratin:

Human hair keratin was extracted according to the Shindai method [7]. The hair (15 g) was mixed with extraction medium, an aqueous solution (400 mL) containing 25 mM Tris, ph 8.5, 2.6 M thiourea, 5 M urea, and 5% 2-mercaptoethanol, at 37°C for 72 h. The mixture was centrifuged at 4500 X g for 15 min, and the supernatant was taken as the keratin solution.

2.3 Quantification of the extracted Keratin:

The quantity of protein in the extracted solutions was determined by the dry weight method. Where, 1 ml of protein solution is weighed and dried in vacuum oven 60°C for 8 hours. The obtained weight difference between the protein before and after drying was taken as the final concentration of the protein. The concentration of obtained Keratin after thorough dialysis is 10 mg/ml.

2.4 Scaffold Preparation:

Two different types of scaffolds were prepared for this study. Pure Gelatin, 12% (w/v) was mixed with deionized water at 60°C for 6 hours. Another scaffold was prepared by mixing Gelatin (3.6 g) and 30 ml of Keratin (10% w/w) for 6 hours at 60 °C by continuously stirring the mixture. The obtained mixture was then allowed to cool at 25°C followed by pre-freezing at -25°C for 24 hours before lyophilizing the scaffolds at -110°C. The freeze-dried scaffolds were then crosslinked with 0.4% Gluteraldehyde at room temperature for 2 hours and then they were washed before being lyophilized once again.

2.5 Royal Jelly Medium:

The medium was prepared with the help of royal jelly (obtained from Hi-Tech Naturals, New Delhi), tender coconut and honey (obtained from commercial store). Initially the medium was prepared with just the two ingredients i.e. 2mg/ml of royal jelly in 100 ml of tender coconut water. The medium was observed to lodge the cells without contamination for more than 3 days, but proliferation was not rapid, hence to supplement the glucose deprived medium, honey was added and the proliferation rate increased. The medium was replaced every 6 hours.

2.6 Cell Culture:

Fibroblast cells were taken from the primary cells that were isolated from the mice. The cells were allowed to grow in a flask containing the cell culture medium (DMEM and 10% FBS v/v) until it became confluent and then it was seeded onto a 96-well sterile cell culture plates. One set of scaffolds of dimension 25 mm^2 were placed on each well and a set of control (without scaffold) was taken and studied simultaneously, the plate was then incubated at 37°C containing 5% CO_2 atmospheric condition. After the cells were seeded onto the scaffolds MTT assay was used to

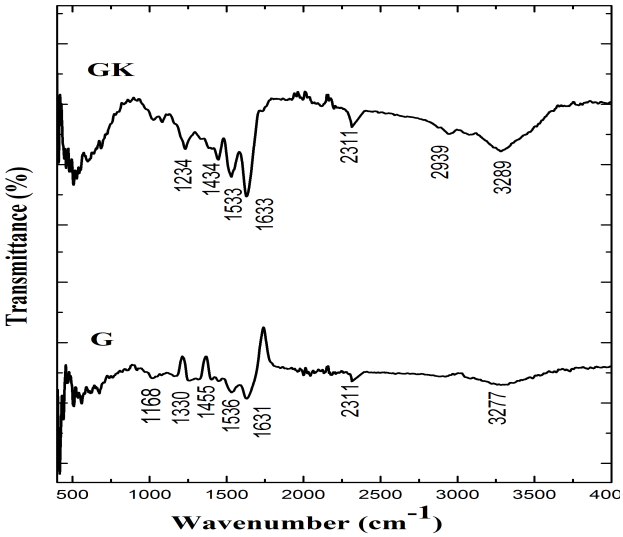
evaluate the proliferation rate of fibroblast cells on all the two scaffolds and their respective OD value was taken on Day 1 and Day 3 with the help of UV-Spectrometer (U-1900 Spectrophotometer, Hitachi High-Tech). The medium was changed every 24 hours without disturbing the scaffolds. The proliferation rate of the cells was observed on Day 1 and Day 3 with the help of Confocal Microscope (ZEN 2010 Carl Zeiss). The same was repeated for another set of scaffolds with the freshly prepared royal jelly cell culture medium by changing the medium every 6 hours.

RESULTS AND DISCUSSION

3.1 FTIR:

The Fourier Transform Infrared (FTIR) (Jasco International Co. /Japan Fourier Transform Infrared Spectrometer Model FTIR-6300 in ATR mode) spectrum of the samples as shown in Figure 2.The characteristic peaks at 1631 cm^{-1} (amide I, C=O bond), 1536 cm^{-1} (amide II bending of N–H bond) and 1455cm^{-1} (stretching of C–N bond in amide III) confirms the presence of Gelatin. The FTIR spectrum of Keratin (GK) (Figure 2) shows a peak at 1633cm-1, 1533 cm-1, and 1234 cm-1 that corresponds to amide I, amide II and amide III respectively. The peak in the range of 670–640 cm−1 of GK can be attributed to the C – S stretching vibrations, but absence of peak between 550 and 500 cm−1 region validate the absence of S–S bond which in turn confirms the presence of reduced (–SH) form of Keratin [8].

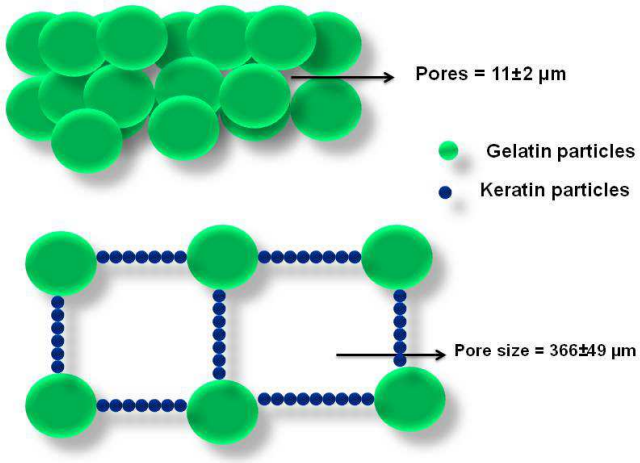
Figure 2: FTIR spectra of G and GK



3.2 Particle Size Measurement:

The particle size of the extracted Keratin was measured with the help of Dynamic Light Scattering (DLS) (ZEN 3600 He-Ne laser (633 nm) at a refractive index of 1.54) and it was found to be 0.174 µm whereas the particle size of 12% Gelatin is 33µm (Table 1). Gelatin particles have the tendency to agglomerate at higher concentrations. However, with the addition of Keratin, the Keratin particles self assembled with each other between each gelatin particles, thereby preventing the gelatin particles from agglomerating to each other. Since the keratin particles also have the tendency of agglomerating (Figure 3) with each other excessively it forms a highly porous structure upon freeze drying.

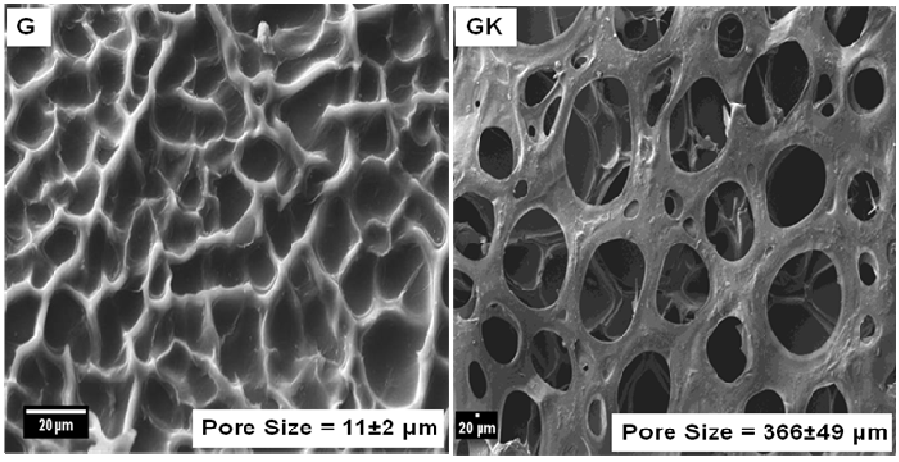
Figure 3: Agglomeration pattern of Gelatin and Keratin molecules



3.3 SEM:

Pure gelatin scaffold showed an average pore size of approximately 11±2 µm (Figure 4), whereas the pore size increases to about 366±49 µm (Figure 4) on the addition of Keratin to the Gelatin. This could be due to much smaller size of the Keratin particles (0.174 µm) than Gelatin particles (33 µm). The otherwise agglomerated form of Gelatin particles gets coupled to Keratin particles, thus separating the Gelatin molecules, thereby increasing the pore size. There is also a significant difference in the porous structure of the two scaffolds; G has shallow pores on the surface whereas GK has an interconnected porous matrix. Scanning electron microscope (SEM) (Carl Zeiss MA 15/EVO 18) was used to observe the surface morphology of the scaffolds. The samples were coated with gold and palladium alloy coating of about 5-10 nm thickness with the help of Sputter coater (Quorum SC 7620) prior to investigation.

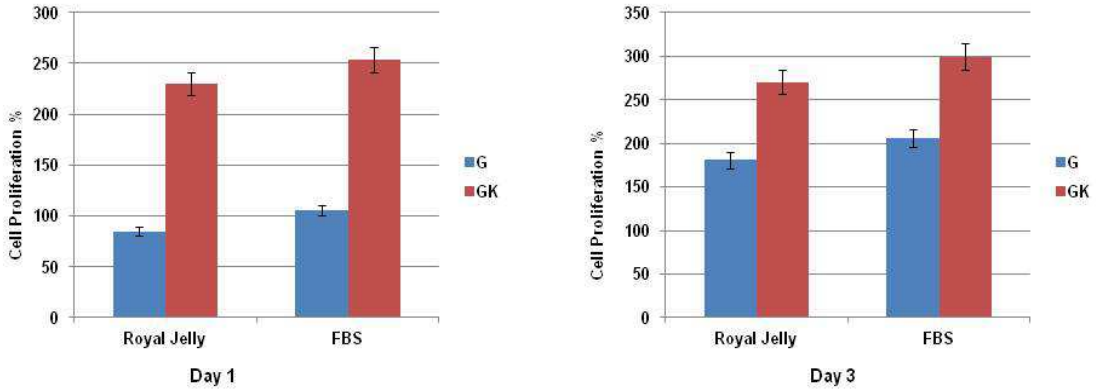
Figure 4: SEM images of G and GK



3.4 Cell Culture:

The MTT assay results (Figure 5) show that the cells show a higher proliferation rate in GK than G. This could be due to the larger pores when compared to G, because of cells generally having a greater affinity towards rough and highly porous matrix.

Figure 5: Cell proliferation of FBS based medium and Serum free natural medium



CONCLUSION

The addition of Keratin has increased the pore size of the scaffolds significantly which has in turn favored the cell attachment and proliferation rate. The difference in cell proliferation rate between the naturally prepared medium and the medium containing FBS is not so significant on Day 3. The cells in the natural medium showed relatively slow growth rate when compared to the medium containing FBS. However, a remarkable quality of the naturally prepared medium was its outstanding anti-microbial efficiency. This could be due to the presence of number of phenolic compounds present in the Royal jelly and Honey that kept microbes at bay.

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