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Comparison of fetal bovine serum and human platelet lysate in cultivation and differentiation of dental pulp stem cells into hepatic lineage cells

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ABSTRACT

The scarcity of organs for liver transplant is a major pressure point of liver transplantation. Hence, generating hepatocytes may provide an alternative choice for therapeutic applications. At present, dental pulp stem cell (SCDs) is an emerging source in regenerative medicine. However, existing protocols for cell culture requires fetal bovine serum (FBS) as a nutritional supplement and may carry the risk of transmitting diseases. Therefore, the present study was undertaken to examine the efficacy of human platelet lysate (HPL) as a substitute for FBS in terms of proliferation and differentiation of SCDs into hepatic lineage cells. The result showed that HPL had displayed a superior effect on the proliferation of SCDs. Next, we induced SCDs into hepatic lineage cells which thrived by initiation and followed by maturation into functional hepatocytes for a total of 21 days. We observed that the gene, protein and its functional profile during this differentiation process reiterated in vivo liver development demonstrating a steady down-regulation of early endoderm markers (GATA4, GATA6, SOX17, HNF4α, HNF3β and AFP) with the up-regulation of hepatic specific markers (TDO, TO, TAT, ALB, AAT, CK18). We also noticed the presence of CK19 suggesting a progenitor population. To ascertain this, we checked for the expression of pluripotent markers and observed that it remained unchanged throughout the experiment period. Our results provide new insights on the ability of SCDs to differentiate into hepatic lineage cells and most remarkably, this can be done in autologous settings whereby both cell source and HPL can be derived from the same donor thus reducing the risk of disease transmission.

1. Introduction

In recent years, the general human population faced a wide range of chronic liver diseases that have led to the deficiency of liver functions. Hence, there is a great necessity for therapeutic intervention to overcome this situation. Previously, allogeneic liver transplantation has been considered as an attractive therapeutic tool for the liver dysfunction [1]. However, this approach has certain boundaries such as serious shortage in organ donor and possibilities of rejection by the host immune system. In observation of these deficits, cell based hepatocytes transplantation has been suggested as the best alternative to cure liver dysfunction since it is simpler, non-invasive and could be cryopreserve for future use [2] Nevertheless, several studies have reported that the direct transplanted hepatocytes cells have lower survival rate and thus could not accomplish enough liver population successfully [1]. Consequently, researches are in search for other effective treatments and cell based therapies have

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appeared to offer a promising alternative for treating liver dysfunction.

In this regards, mesenchymal stem cells (MSCs) which are known as a versatile and a 'universal' cell can be used for the treatment of several human diseases such as liver related diseases. Likewise, dental pulp stem cells from extracted deciduous teeth (SCDs) were shown to putatively form post natal stem cells that are capable of self-renewal, highly proliferative and also multipotent [3]. Previously, we as well as other researchers have shown there are tremendous differentiation potentials of SCDs such as neuronal differentiation [3], beta cell lines [4], and cardio differentiation [5]. Additionally, recent studies have found that SCDs may undergo hepatocyte differentiation if given veracious microenvironment for induction [6,7].

All the above mentioned research portrayal of SCDs were customarily conveyed in ex vivo conditions that contain basal medium supplemented with FBS which is a crucial nutrient for cells. However, one serious concern on the usage of FBS is the risk of animal orientated viral and pathogens. Moreover, the variation of composition in lot-to-lot collection of FBS has resulted in phenotypical differences [8]. From the point of view of animal ethics, the collection of FBS has caused great grief among the animal lovers as it involved the killing of prenatal cows as well as calf fetus [9]. Since numerous concerns have been put forth pertaining to the usage of FBS, consequently, a paradigm shift to animal serum-free alternatives is promoted by regulatory authorities and research communities in general. A chemically defined xeno-free media could be the preferred solution. However, such a formulation that allows for both isolation and expansion has not been fully achieved thus far. Further, making a chemically defined media as well as FBS itself ultimately could be an obstacle if the cells go for a large scale clinical stage [10].

Thus, initiation of human-based supplement, explicitly HPL, has been introduced increasingly in stem cell therapy as a potential surrogate to FBS. The components that reside within the HPL are; platelet derived growth factors, basic fibroblast growth factors, vascular endothelial growth factors, insulin growth factors and transforming growth factors-β are known to serve as cell culture growth factors [11]. Among the persuasive mediator released from platelet are adhesive protein, coagulant factors, mitogen, protein inhibitors and proteoglycan [12]. Hence, HPL may replace FBS in many cell culture systems. Despite this, the current body of literature on the usage of HPL in stem cells are heavily focused on its proliferation capacity and little is known on the differentiation capacity. We have previously reported that HPL permits extensive proliferation of SCDs for clinical application [10]. Here, we investigated the trans-differentiation potential of SCDs exposed to HPL media toward hepatic lineage cells. The hepatic lineage cells generated from SCDs which had been exposed to HPL media offer an alternative source of hepatocytes which will provide great advantages in liver disease treatments especially in autologous settings whereby both cell source and HPL can be derived from the same donor thus reducing the risk of disease transmission.

2. Materials and methods

2.1. Isolation of cells

This study was conducted under ethical approval from the Medical Ethics Committee, Faculty of Dentistry, University of Malaya [DF CD 0907/0042 (L)]. SCDs cultures were obtained from four young donors ($n = 4$; age 3–9 years old) as previously described [3,4]. Briefly, root surfaces were cleaned with Povidone-iodine (Sigma Aldrich, St. Louis, MO, USA; http://www.sigmaaldrich.com) and the pulp were extirpated within 2 h post-extraction and processed. The pulp tissue was minced into small fragments prior to digestion in a solution of three mg/mL collagenase type I (Gibco, Grand Island, NY, http://www.invitrogen.com) for 40 min at 37 °C. After neutralization with 10% FBS, the cells were centrifuged and seeded in culture flasks.

It should be noted that human hepatocellular carcinoma cell line (HepG2, ABM, Biorev) was used as a positive control cell lines in the present study. These cells are highly differentiated and display many of the genotypic features of normal liver cells [13].

2.2. Human platelet lysate (HPL)

HPL was prepared as described by our group previously [10] in current good manufacturing practice (cGMP) condition. We also reported that HPL is free from animal origin, has been characterized, and passed the quality control test in terms of endotoxin level, mycoplasma, pH and sterility prior to the SCDs expansion and differentiation usage.

2.3. In vitro expansion of SCDs culture

For expansion of the SCDs culture, culture media was prepared using basal media of Knockout DMEM (Invitrogen) supplemented with 10% Australian characterized FBS (Hyclone, MA, USA, http://www.thermofisher.com), 0.01× Glutamax (Invitrogen) and 0.5% penicillin/streptomycin (Invitrogen). Since the amount of seeding materials is so small, taking the chance to lose the materials by adapting to new ingredients is risky. Therefore, we initially used the FBS to set the ball rolling since that is the commonly used nutrient in any media composition for culturing. At passage 1 (P1), the cells were divided into 2 groups, we retained the FBS in one group and introduced HPL supplemented with 0.001 mL of heparin (Heparinol) to the second group. The cells were cultured for 5 passages (P1–P5). We allowed the cells to acclimatize to HPL condition at least for one passage before we started with the differentiation process at P3 whereas other basic experiments except for growth kinetics were conducted at P5.

2.4. Growth kinetics

In this procedure the proliferation rate was determined by plating 5000 cells/cm^2 of each SCDs into T25 cm^2 culture flasks (BD Pharmingen). Three replicates were performed for each passage. Cells were detached by trypsinization, after reaching confluency of 90%. Cells were then counted and assessed for viability by means of trypan blue dye exclusion before splitting the cells into the next passage. Cells were re-plated for subsequent passages and a total of 5 passages were studied in this experiment. Growth kinetics was analyzed by calculating population doubling (PD) time. The PD time was obtained by the formula:

$$\mathrm{PDT} = \frac{\log_2(\mathrm{time})}{\log(\text{initial no. of cell} - \text{final no. of cell})}$$

2.5. Colony forming unit (CFU) assay

The colony forming unit (CFU) assay was determined by replating 100 cells in 35 mm dish (BD Bioscience) followed by 14 days of culture at 37 °C with 5% CO_2. Then, the cells were rinsed twice from growth media using Dulbecco's Phosphate Buffered Saline (DPBS, -Ca^{2+}, -Mg^{2+}; Invitrogen) and fixed with 100% methanol (Mallindkrodt, Hazelwood, USA, http://pharmaceuticals.covidien.com) for 20 min at room temperature (RT), followed by 3% crystal violet (Sigma Aldrich) staining. Next, the blue stain was