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Industrial-scale processing of activated platelet-rich plasma from specific pathogen-free pigs and its effect on promoting human hair follicle dermal papilla cell cultivation

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ABSTRACT

Due to the controversial nature of fetal bovine serum (FBS) production and safety concerns, there is a major interest in finding a potential FBS replacement, such as human platelet-rich plasma (PRP). However, traditional PRP production is largely limited by yield and is unsuitable for large-scale production. In this study, our first aim is to optimize the porcine PRP production protocol, and render it capable of manufacturing a large amount of porcine PRP that is suitable as an FBS replacement. Additionally, our secondary aim is to determine the effects of porcine PRP on human follicle dermal papilla cells (HFDPCs). In our process, the platelets are separated and concentrated via small- (with a capacity of 2.4 L/h) or large-scale preparations (with a capacity of 200–500 L/h). The porcine PRP is activated using a combination of collagen, thrombin, and calcium chloride, resulting in three distinct PRP formulations. Growth factor levels and stabilities of PRP under different treatments were measured using ELISA, MTT and real-time PCR assays. Our results showed that porcine PRP has higher growth factor levels than FBS, and is stable during freeze-drying and surfactant treatments, whereas HFDPCs treated with freshly prepared or freeze-dried PRP showed no significant difference in proliferation compared to FBS. In conclusion, our study is the first to confirm the commercial viability of porcine PRP as an FBS replacement and hair growth promoter.

1. Introduction

The use of animal serum is still needed for successful *in vitro* cell culture because serum contains high concentration of nutrition and growth factors [1]. Currently, fetal bovine serum (FBS) is the most commonly used serum additive due to its versatility and ability to support the growth of various cell types. The use of FBS, however, is rife with controversies [2]. From an ethical standpoint, FBS production has been considered to cause undue pain to fetal calves [3]. From a scientific standpoint, the composition of FBS is largely undefined and has significant batch variations [3–5]. Most importantly, however, are the safety concerns that using xenogeneic serum may lead to the possibility of disease transmission and immune reactions [6]. Therefore, there is a focus on finding an alternative to FBS.

Platelet-rich plasma (PRP) is a preparation of concentrated blood platelets suspended in plasma [7]. As such, they are rich in various growth factors and cytokines that have been shown to aid in wound healing and angiogenesis [8]. These proteins can be released through degranulation for practical purposes and targeted delivery [9]. Depending on the degranulation method, platelet lysate (PL) and activated PRP have been derived from PRP. PL uses freeze-thawing to lyse the platelets and release the proteins [10], whereas activated PRP uses agonist solutions such as thrombin or calcium chloride [11,12], that mimic the natural coagulation process. Both PL and activated PRP have been successfully used in mesenchymal stem cell culture, highlighting their potential as a viable FBS replacement [10,13–15]. Nevertheless, human PRP is impractical for industrial production, as it is severely limited by supply and concerns over disease transmission [16,17]. In our previous study, we collected and produce porcine PRP from specific

Abbreviation: CDE, cocamide DEA; CPB, cocamidopropyl betaine; CPD, citrate phosphate dextrose solution; FBS, fetal bovine serum; HFDPCs, human follicle dermal papilla cells; L, large scale; PBS, phosphate buffered saline; PL, platelet lysate; PRP, platelet-rich plasma; QPCR, real-time PCR; S, small scale; SDS, sodium dodecyl sulfate; SPF, specific pathogen-free.

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pathogen-free (SPF) pigs to reduce the risk of disease transmission. We demonstrated that porcine PRP contained high concentration of growth factors, such as IGF-1, FGF7/KGF, PDGF-AB and TGF-β, and porcine PRP can be as an FBS replacement used in mesenchymal stem cell culture [18]. Porcine PRP has the distinct advantage of further reducing cost and the environmental impacts of the pig industry. However, the current method of porcine PRP production is labor-intensive and has relatively low yield, making it unsuitable for commercial production. Thus, it is necessary to improve the protocols for large-scale production of porcine PRP and to make them suitable for commercial production.

Although otherwise harmless, alopecia, more commonly known as hair loss, has been known to cause significant distress in patients [19,20]. In a healthy individual, the hair follicle is responsible for the hair growth cycle, from anagen (growth) to catagen (degeneration) and telogen (resting) and then back to anagen [21]. A particular type of cell in the base of the human follicle, known as the human follicle dermal papilla cells (HFDPCs), is of particular importance, as it controls the mechanisms responsible for the hair growth cycle [22–24]. Under alopecic conditions, changes in the hair cycle result in shorter anagens and longer telogens, which only worsen as the condition progresses, eventually resulting in a permanent telogen phase [25]. Common treatments for alopecia include Minoxidil and Finasteride, which work by prolonging anagen through promotion of HFDPC survival [26–28]. However, as these treatments are unable to form new hair follicles and are accompanied by significant side effects, there is interest in finding alternative treatments for alopecia [29,30]. Although activated PRP has previously been used to aid in wound healing and post-operative recovery, activated PRP has also recently been used to treat alopecia [31]. PRP-treated follicle transplants yielded improved results, and the application of PRP has been shown to promote hair growth as well [31–33]. According to these literatures, we speculated that PRP would be a good choice as an additive to hair care products aiding to promote hair growth. Importantly, for the safety consideration, PRP products serve for external use would be a suitable application.

Our objectives are thus threefold: to develop manufacturing protocols capable of large-scale commercial production of porcine PRP, to assess the porcine PRP product as a viable FBS replacement and to assess the ability of porcine PRP product to promote the growth of HFDPCs.

2. Materials and methods

2.1. Blood collection, centrifugation and PRP preparation

Animal test was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of Chinese Culture University (CCU10003). Fresh porcine blood was collected and pooled from around five hundred SPF pigs at the local slaughterhouse in Pingtung County, Taiwan and was anticoagulated using a citrate phosphate dextrose solution (CPD) at a 1:7 ratio; the sample were mixed well and kept chilled on ice prior to centrifugation. Immediately after collection, the anticoagulated blood was centrifuged using 2 distinct machines to create the small-scale (S) preparation and the large-scale (L) preparation of PRP.

The S preparation (with a capacity of 2.4 L/h) was centrifuged using a Beckman Coulter Allegra™ 6 laboratory benchtop centrifuge (366,802, Beckman–Coulter, Fullerton, CA) and using the double-spin method. First, the anticoagulated blood was placed into 500 ml tubes and centrifuged at 1000 × g for 10 min to separate the red blood cells and the plasma. After the first centrifugation, the plasma was collected (30–45% of total blood volume) to further centrifuge at 3000 × g for 10 min to separate the buffy coat and the plasma. The resultant buffy coat and plasma were separately collected. Then, the collected buffy coat was resuspended in the plasma and adjusted to a concentration of 500 platelets/μl using an Auto Hematology Analyzer.

Table 1
PRP formulations.

PRP formulation	Collagen	Thrombin	$CaCl_2$
Co + T − Ca−	1000 μg/ml	1 U/ml	0.1%
Co + T + Ca−	1000 μg/ml	10 U/ml	0.1%
Co − T + Ca−	10 μg/ml	10 U/ml	0.1%

The L preparation (with a capacity of 200–500 L/h) was centrifuged using an industrial-scale continual flow centrifuge (S-VT No. 6, Chuan Tai Iron Factory, Taiwan) at 25,000 × g. This process resulted in two different phases of centrifuged blood, the light phase and the heavy phase. The light phase was collected, and its platelet count was adjusted to 500 platelets/μl through dilution with phosphate-buffered saline (PBS). The resultant S and L PRP were then kept chilled at 4 °C until further use.

2.2. Agonist preparation

Collagen was extracted from pig skin using a previously described protocol [34], lyophilized technical grade bovine thrombin was purchased from Biopharmlaboratories (SKU 91–010, Utah), and calcium chloride was purchased from Katayama Chemical (F1350, Osaka, Japan). Collagen was first dissolved in 0.1 M acetic acid, and all above reagents were finally dissolved in PBS. The three best formulations of agonist, based on a previous study [18], were used for the current study (Table 1).

2.3. Activation

Each agonist formulation was activated using both the small and the large scale PRP. PRP was mixed with the agonist solutions at a ratio of 5:1:1:1 for each agonist (PRP: collagen: thrombin: $CaCl_2$) and incubated at 37 °C for 30 min in a shaking water bath (SB-301, Double Eagle Enterprise, Taiwan). Then, the incubated PRP was heat-inactivated at 56 °C for 30 min with continued shaking. Finally, the heat-inactivated PRP was centrifuged at 15,000 x g for 10 min, and the supernatant was collected and sterilized through a 0.22 μm filter to remove contaminants and debris. A total of 6 activated PRP samples were produced from the process: (1) PRP Co+T−Ca− S, (2) PRP Co+T+Ca− S, (3) PRP Co−T+Ca− S, (4) PRP Co+T−Ca− L, (5) PRP Co+T+Ca− L, and (6) PRP Co−T+Ca− L, which were either designated for further use or stored at −80 °C for long-term storage.

2.4. Hemoglobin

Determination of hemoglobin according USP method, absorbance of the P-PRP and serum sample using a spectrophotometric cell of 1 cm path length at of absorbance at 576, 623, and 700 nm and using water as a blank. Calculate the concentration of hemoglobin in mg/dl:

$$(Abs_{576} \text{ X } 115) - (Abs_{623} \text{ X } 102) - (Abs_{700} \text{ X } 39.1) = \text{conc. (mg/dL)}$$

2.5. Freeze-thaw cycles

The finished PRP products were subjected to further freeze-thaw stress by cycling between −80 °C and 37 °C either 5 or 10 times. Five milliliters of samples were first frozen at −20 °C for 2 h in sterilized glass bottles. The samples were further frozen at −80 °C for 2 h. The frozen samples were then put into a freeze

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