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Original Article

Optimization of Animal Sera-Free Culture Condition for Generation and Expansion of Human Cardiosphere-Derived Cells

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Abstract:

Background: Preclinical studies have introduced cardiac stem/progenitor cells (CSCs) as a promising cell candidate for cell therapy of heart diseases. CSCs can be isolated from myocardial biopsies using various protocols, expanded *in vitro* and transplanted back to the patients. One of the most important issues regarding the clinical usage of cells is the choice of suitable humanized culture supplements to replace commonly used animal-derived products including fetal bovine serum (FBS) or fetal calf serum (FCS).

Methods and Materials: In order to find the optimal FBS substitute, human myocardial samples were cultured as explants in media supplemented with one of these different blood products: FBS, human serum (HS), human plasma (HP) or platelet lysate (PL). The out-grown cells were cultured in suspension to generate cardiospheres and then plated to expand as cardiosphere-derived cells (CDCs). The effect of culture media on the process of CDC generation and culture was evaluated in terms of morphology and cell growth.

Results: Among the examined humanized agents, CDCs were only generated and expanded in medium supplemented with HS. Furthermore, they had normal karyotype and expressed CSC associated surface markers but not endothelial and hematopoietic markers. Moreover, cultured CDCs in HS inhibited the proliferation of induced lymphocytes *in vitro* which might highlight the immuno-modulatory feature of these cells.

Conclusion: Taken together, our data exhibited the superiority of HS compared to other tested human blood products for CDC culture which can be suggested for cell culture set up of cardiac clinical studies.

Key words: Progenitor Cells, Culture Medium, Human Serum, Cell Therapy

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Introduction

During the last decades, because of changes in human life-style, cardiovascular diseases (CVDs) has converted to one the leading causes of death worldwide and therefore, are considered as an important global health issue (2, 3). Today, distinct branches of regenerative medicine especially cell therapy have been introduced as alternates of commonly used treatments for CVDs (4-6). Among various suggested cell types obtained from non-cardiac or cardiac origins, cardiac stem/progenitor cells (CSCs) exhibited more regenerative capacity, improved cardiac performance, might have immuno-modulatory and paracrine effects and lacked safety concerns of tumorigenicity or arrhythmia after transplantation into the damaged heart (7, 8). CSCs are cardiac-committed cell types with proliferation and differentiation capacities which can be isolated from myocardial biopsies, cultured *in vitro* and transplanted back to the patients (8, 9).

There are different methods introduced for isolation of resident CSCs from cardiac tissue samples mainly based on cell surface markers or adhesion characteristic. For example, myocardial biopsies can be cultured as explants to provide a suitable culture system for expansion of CSCs. We and others have shown that suspension culture of explant-derived cells could enrich its stem cell population called cardiospheres (9-12). In order to have sufficient cell count required for cell transplantation, *in vitro* culture and expansion of cardiosphere-derived cells (CDCs) are as prerequisites. To promote cell adhesion, culture and proliferation, serum supplements which are rich in growth factors and hormones are added to the culture media; fetal bovine serum (FBS) or fetal calf serum (FCS) are commonly used in this regard (13). However, the usage of animal-derived products in clinical studies may introduce microbial contaminations as well as batch to batch variations and induce immunological reactions (13-15). Furthermore, providing the clinical grade xeno-derived materials are too expensive. Hence, identification of suitable alternates for development of xeno-free culture media may overcome these challenges and lessen the cost of cell manufacturing procedure beneficial for patients.

In order to find the most efficient replacement for FBS, different humanized supplements including human serum (HS), human plasma (1) and platelet lysate (PL) were used in culture media as alternates. Our findings indicated that HS was the superior substitute for FBS which preserved the characteristics of CDCs. Furthermore, cultured CDCs in HS suppressed the proliferation of stimulated lymphocytes.

Materials and Methods

Sample sources

Heart tissue samples were myocardial biopsies which were obtained during heart transplantation surgeries in Shahid Rajayi Hospital (n=10, comprised of 8 male and 2 female samples). According to the standard therapeutic protocol for the patients with a transplanted heart, an endomyocardial biopsy sampling should be performed with the related informed consent. As well, an additional informed consent was obtained regarding the investigational usage of a small part of their cardiac samples. Biopsies approximately 3 mm^3 were transferred to culture laboratory in cold transfer media containing IMDM (Sigma-Aldrich; 13390), 20% FBS (Gibco; 10270-106) and 5% penicillin/streptomycin (Gibco; 15070-

063). The study was performed based on the ethical protocols approved by Institutional Ethical Committee of Royan Institute.

Human serum (HS), human plasma (HP) and platelet lysate (PL) preparation

Human blood samples with AB blood type from healthy volunteers without any known acute or chronic diseases were collected to prepare HS in the absence of anti-coagulant agents. For serum isolation, blood samples were stored at room temperature for 3 hours to allow clot formation and then were centrifuged at 3000 g for 30 minutes to remove erythrocytes and coagulum contents. HS was heat inactivated by incubation at 56°C for 30 minutes. HP was obtained from fresh adult and cord blood samples which the latter was stored in the public cord blood bank of Royan Institute. Blood samples were collected in plasma tubes containing anti-coagulant agents, inverted several times to be mixed and then centrifuged. The supernatant was carefully transferred into new tubes. Similar to HP, PL preparation was performed using stored samples in the public cord blood bank of Royan Institute as previously described (1). After testing the microbial contamination including HIV-I/II, HCV, HBV, CMV, and HTLV-I/II, the negative samples were aliquoted in 50 ml sterile tubes, labeled and cryopreserved at -20° C. Thawed samples were filtered after adding to the culture media.

Cardiosphere-derived cells (CDCs) generation and culture

Human myocardial samples were subjected to cell isolation procedure as previously described with some modifications (9). After washing twice with cold DPBS containing 5% penicillin/streptomycin, the blood clots and the remained visible parts of vessels were removed from myocardial biopsies. Heart tissue samples were cut into small pieces (1 mm^3) and then were cultured as explants on fibronectin (Sigma-Aldrich; F0635) -coated plates. Explant culture medium was supplemented with either FBS 10%, HS 10%, HP 10%, or PL 10%. Some days after culture, a fibroblast layer was grown over which phase bright cells were migrating. Mild enzymatic incubation at room temperature was used to collect these migrating loosely attached cells. Collected cells were cultured on ultra-low attachment plates (Corning) in cell culture medium including IMDM, serum supplements, 2mM l-glutamine (Gibco; 25030-024) and 1% penicillin/streptomycin to generate spheroids called cardiospheres. Large enough cardiospheres were cultured on fibronectin-coated plates to grow adherently as CDCs. Every explant was passaged 3 or 4 times for cardiosphere generation. To assess the quality of isolated cells, flowcytometry analysis, doubling time measurement and karyotyping as well as morphological observations were used.

Flowcytometry analysis

To evaluate cell surface antigen distribution, flow cytometery analysis was performed. Candidate cell surface markers were CD90 (Dako; F7274), CD117 (MiltenyiBiotec; 130-091-734), Sca-1 (eBioscience; 12-5981-81), CD31 (BD Pharmingen; 555445), CD34 (BD Pharmingen; 550619) and CD45 (Dako; F0861). To assess the percentage of positive cells for each marker, 5×10^4 CDCs at passage 4 were used (n=2). Data obtained by BD FACScalibur (BD Biosciences, San Jose) system was analyzed with WinMDI software 2.9.

Doubling time measurement

To measure the essential time for cell division, the population doubling time (PDT) was calculated as previously described (9). Briefly, CDCs at passage 4 were cultured at a seeding density of 5×10^4 cells/cm^2 in 4-well plates and counted at days 2 and 4 using trypan blue exclusion method and the PDT was measured using the following formula:

$N = N_0 2^{t/T}$,

N = Daily cell count

N_0 = Initial cell count

T = PDT.

Cytogenetic assessment

CDCs at passage 4 were undergone karyotype analysis (n=8). Four T75 cm^2 cell culture flasks with 60-70% density were used for each sample. Cells were treated with 30 µg/ml Colcemid (Gibco; 15210-057) for 45 minutes in cell culture incubator at 37° C. Cells were detached using trypsin/EDTA solution and the cell pellet was treated with KCl 75 mM for 15 minutes. For fixation, ice cold freshly prepared methanol:glacial acetic acid (3:1) was used and washed cells were spread on clean slides drop-wisely. Air-dried slides were used for staining to analyze the metaphase spreads.

Mixed lymphocyte reaction (MLR)

CDCs were pretreated with 10 µg/ml Mitomycin C (Sigma-Aldrich; M0503) for 2 hours at 37° C and cultured on round bottom 96-well plates at a density of 10^5 cells/well. Human peripheral blood mononuclear cells (PBMCs) were isolated from two healthy HLA-mismatched donors' bloods using Ficoll density gradient (Sigma-Aldrich; F5415) based on the manufacturer instruction. PBMCs layers were cultured in αMEM (Sigma-Aldrich; M4526) containing 10% FBS, 1% penicillin/streptomycin, 2mM l-glutamine and 1% nonessential amino acids (Gibco; 11140-035) for an overnight to let the monocytes attach the plate. The next day, the suspending lymphocytes were collected, washed and one sample was treated with 25 µg/ml Mitomycin C for 45 minutes at 37° C as stimulator group (S). The responder lymphocytes (R) were stained with Carboxyfluorescein succinimidyl ester (CFSE) cell proliferation kit (Invitrogen; C34554) according to the manufacturer protocol. Briefly, cells were suspended in PBS/0.1% BSA containing 10 µM CFSE and incubated at 37° C for 10 minutes. Then cold αMEM was added and placed at 4° C for 5 minutes. After washing the cells, S and R lymphocytes were co-cultured with each other (R+S) and or with CDCs (R+S+CDCs) for 3 or 4 days and the proliferation rates were calculated based on the CFSE dilution analyzed by flowcytometry system for three experimental replicates. The R lymphocytes cultured alone were served as negative control group.

Statistical analysis

Data is obtained from at least two independent replicates and presented as mean ± standard deviation. To analyze the significance level between groups, student's t-test was used and the data with p-value≤0.05 was determined as statistically significant.

Results

Cell culture groups

Myocardial biopsies were minced and cultured as explants and the out-grown cells were used for cardiosphere and then CDCs generation. In order to find the best humanized serum substitute for FBS,

explants were divided into 4 main groups, each one cultured in medium supplemented with either FBS 10% (control group, group 1), HS 10% (group 2), HP 10% (group 3), or PL 10% (group 4) (Figure 1). Being confluence enough, loosely attached out-grown cells were isolated from explants and were cultured in suspension condition to purity stem cells grown as cardiospheres. In the last step, cardiospheres were cultured adherently in the same culture media to have sub-cultures of them called CDCs.

The effect of serum supplements on explant and cardiosphere culture

In the first step, to compare the effect of different medium supplements on explant culture and CDCs isolation, some phenotypic criterion were evaluated including explants adhesion to the plate, explants quality, minimal essential time for cell growth, quality of generated cardiospheres, the number of explant passages, and the number of CDC passages (Table 1). Explants from viable biopsies should attach to the culture plate during the first culture hours; they were attached in HS group the same as FBS control group, but some explants in HP groups were detached after the first medium refreshment and no explants were remained attached to the plates after some media refreshments in PL group. The attached explants in FBS, HS and HP groups had typical tissue morphologies (brown live tissues) while in PL group most of the explants were degraded (Figure 2 A); so, the PL group was removed from the experiment. Approximately one week after culture, fibroblast-like cells were out-grown from explants in FBS and HS groups while this time was doubled for cultured explants in HP group (Table 1).

Cardiosphere generation was performed for FBS, HS and HP groups; the quality and the density of generated cardiospheres in FBS and HS groups were the same, while in HP group, less cardiospheres were generated compared to HS and FBS groups. Of note, cultured spheroids in FBS and HS groups were attached to tissue culture plates after plating and grown as CDCs while explant-derived cardiospheres which were cultured in HP-supplemented medium could not attach after plating (Table 1, Figure 2 B); hence, medium supplemented with HP was not suitable for CDC formation and this group was also removed from further experiments.

Characterization of cultured CDCs

As explained in the previous part, HS was the only FBS substitute suitable for CDCs generation and culture among our tested groups. In order to identify the cell characteristics in HS group, doubling time as an index of cell growth, karyotype and expression of surface markers were evaluated. As Figure 3 A shows, CDCs cultured in both FBS and HS groups had around 30-40 h measured doubling times (the difference was not statistically significant). Moreover, to determine whether medium supplementation with HS would cause chromosomal abnormalities in the cultured CDCs, cytogenetic analysis was performed. Interestingly, no chromosomal abnormalities were detected in karyotype of CDCs in HS group at passage 4 (Figure 3 B). Furthermore, to compare the characteristics of grown CDCs in HS-supplemented medium with cultured CDCs in FBS group, the expression of cell surface antigens were analyzed. As Figure 3 C shows, CDCs in HS group the same as CDCs in FBS group expressed surface markers associated with cardiac stem cells including CD90, CD117 and Sca-1 and were negative for endothelial and hematopoietic associated markers such as CD31, CD34 and CD45.

Table 1. Timing and quality of explants and CDCs cultured in media supplemented with different FBS substitutes.

Medium supplement	Explants adhesion	Explants quality	Essential time for cell growth	Quality of cardiospheres	The number of explant passages	The number of CDC passages
FBS	+	Live tissue	7-10 days	Typical cardiosphere morphology	Three times	Three times
Human Serum	+	Live tissue	7-10 days	Typical cardiosphere morphology	Three times	Three times
Human Plasma	+/-	Both live and degraded tissues	14-16 days	Small cardiospheres, without proper growth	-	-
Platelet Lysate	-	Degraded tissue, full of debris	-	-	-	-

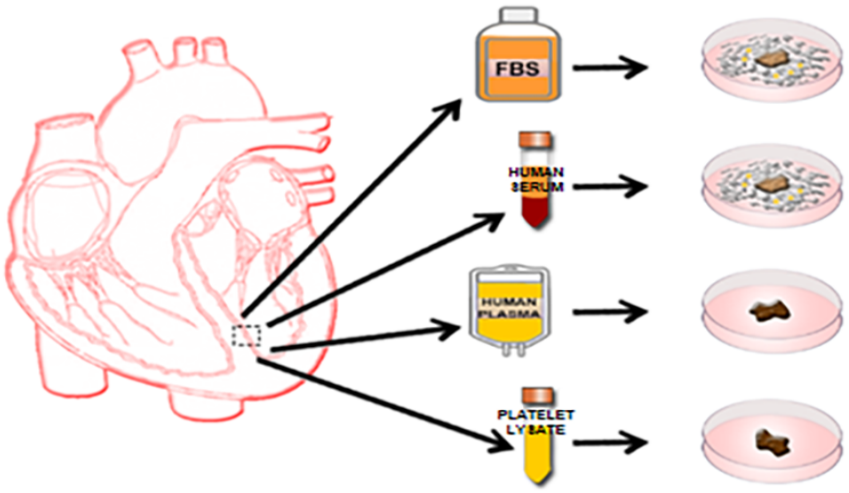

Fig. 1. Schematic diagram showing our study groups. Myocardial biopsies obtained during heart transplantation surgeries were cultured as explants in four different culture media supplemented with FBS, human serum (HS), human plasma (HP) and platelet lysate (PL). Out-grown cells from explants were harvested and cultured in low-attachment plates to purify stem cells which were grown as spheroids. Cardiospheres were then plated to proliferate and generate cardiosphere-derived cells (CDCs).

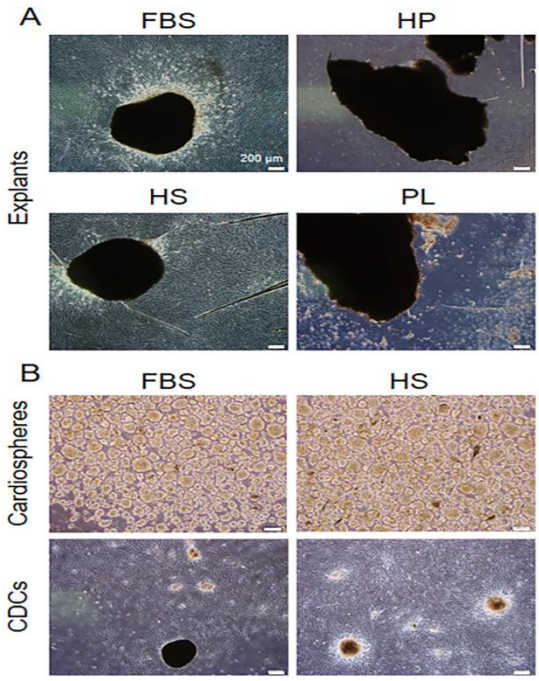

Fig. 2. Cultivation of explants in different media supplemented with FBS or humanized substitutes. (A) The morphology of explants in four different culture groups. Cells were greatly out-grown from explants in FBS and HS groups but not in HP and PL groups. (B) The morphology of generated cardiospheres and CDCs in FBS and HS groups. Cardiospheres were plated and grown adherently to generate CDCs. Scale bars present 200 µm.

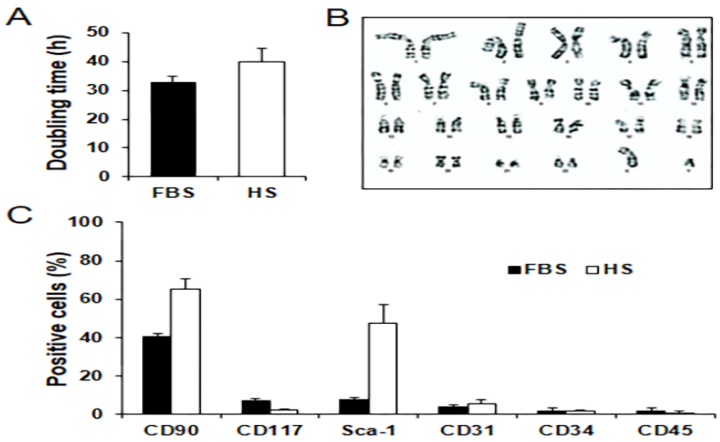

Fig. 3. Characterization of CDCs cultured in medium supplemented with HS. (A) Doubling time measurement of CDCs at passage 4 cultured in HS group as well as FBS group as a control. The measurement showed a partial increase in doubling time of CDCs in HS group compared to FBS group although this difference was not statistically significant. (B) Cytogenetic evaluation of CDCs in HS group at passage 4 which exhibited their normal karyotype. (C) Analysis of cell surface markers associated with CSCs as well as endothelial and hematopoietic cells for CDCs in FBS and HS groups. All data are presented as mean ± standard deviation.

Immunological reaction assessment

In order to determine whether cultured CDCs in HS-supplemented medium would stimulate the immune response, the direct effect of these cells on lymphocyte proliferation was examined by MLR assay. For this regard, to trigger the proliferation of R lymphocytes, they were co-cultured with HLA mismatch S lymphocytes and then this cell mixture was added to CDCs (R+S+CDC group). As control groups, R alone and R+S were used. The morphology of lymphocytes and CDCs before and three days after co-culture is presented in Figure 4 A. To demonstrate the most suitable ratio of CDCs and R mixture, the proliferation of R was studied for three different concentrations of CDC:R (1:10, 1:5 and 1:1) at days 3 and 4 post co-culture. As lymphocytes proliferate in response to stimulation, the intensity of CFSE decreases and shifts to the left side of flowcytometry histogram which shows different generations of lymphocytes after cell divisions. Flowcytometry analysis of CFSE showed no proliferation in R alone group. However, the analysis of cell percentage in the left side of histograms (the last generation of cells) showed that more than 50% of R cells were proliferated due to co-culture with S group (R+S) while R proliferation was significantly inhibited in all R+S+CDC groups (Figure 4 B); hence, CDCs not only did not induce lymphocyte proliferation, but also modulated the immune response of lymphocytes. Moreover, it was indicated that the most inhibition of R proliferation was happened in 1:1 ratio of CDC:R. In addition, three days co-culture seems to be enough for studying the immunological features of CDCs.

Discussion

Currently, most cell culture techniques are based on the usage of animal-derived agents such as sera, enzymes and growth factors which raise several safety concerns including infection and immunological reactions. The undefined nature of xeno-derived sera such as FBS and the resultant uncontrollable impacts on cell characteristics highlighted the need to find FBS substitutes for clinical applications (16). In agreement, it is reported that FBS-derived exosomes affected the behavior of explant-derived CDCs including proliferation and spheroid formation (17). Therefore, in the current study, it was tried to examine the impact of FBS replacement with human blood derived products on the culture and expansion of human CDCs *in vitro*. In order to identify the most suitable FBS alternate with clinical applications, HS, HP and PL were studied. The outcome of preclinical and clinical studies revealed that myocardial-derived CSCs are great cell candidates for cell therapy of heart diseases (7). We and others tried to isolate different sub-populations of CSCs to compare them based on characteristics and regenerative potential (9, 18, 19). Our results unveiled the superiority of CDCs compared to other sub-populations (9). Therefore, CDCs were used in the current study to investigate the best FBS substitute for their culture. Myocardial biopsies were cultured in different media supplemented with humanized FBS alternates and the process of explant culture and CDC isolation was precisely monitored. Our observation determined that among the studied human blood products, HS the same as FBS was more compatible for explant culture and CDC generation. Our data was in agreement with other studies which highlighted the superiority of HS compared to other supplements for cell culture (20-22).

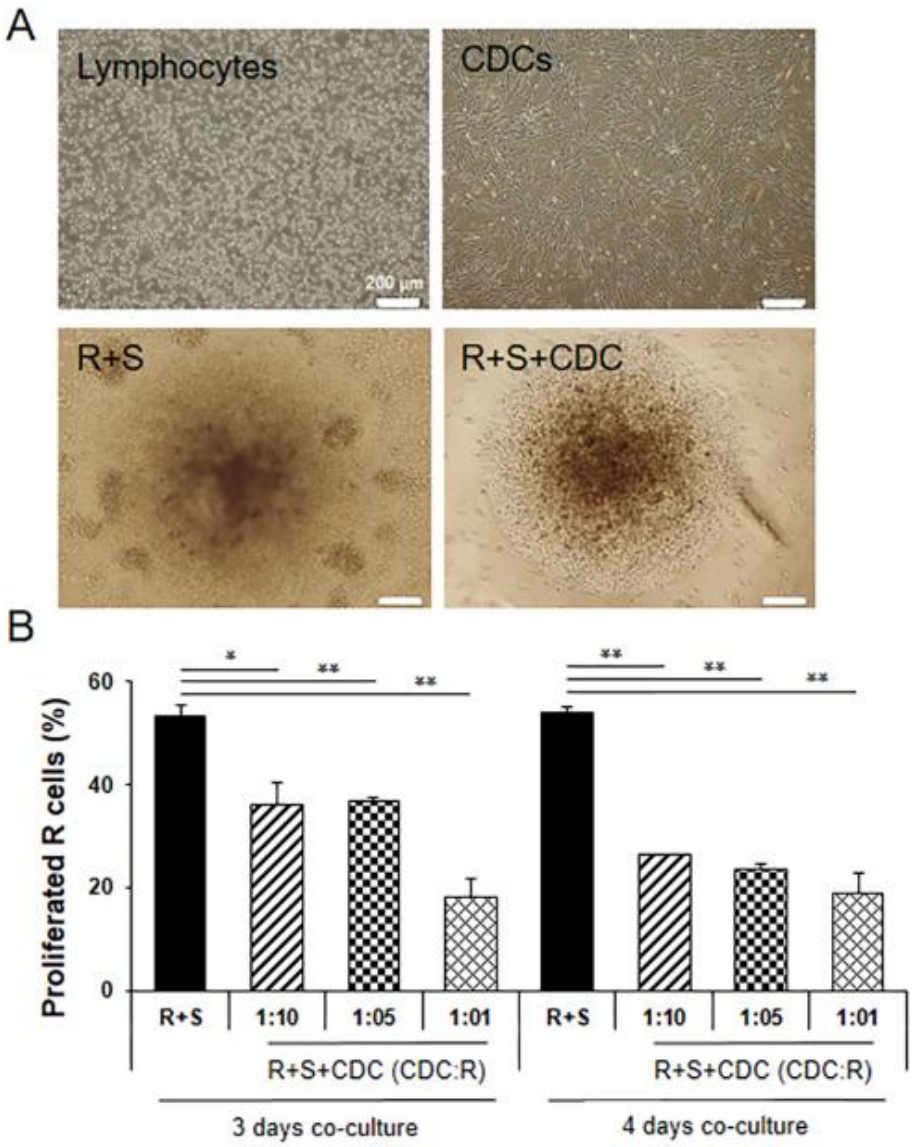

Fig.4. Immunological reaction of CDCs cultured in medium supplemented with HS. (A) Phase contrast images of lymphocytes, CDCs and co-culture groups (at day 3 after co-culture) for MLR assay. R: responder lymphocytes and S: stimulatory lymphocytes. Scale bars present 200 µm. (B) Proliferation analysis of CFSE-labeled R cells in response to co-culture with allogeneic S or S+CDCs after three or four days. It was exhibited that CDCs significantly decreased the R proliferation which was stimulated as a result of co-culture with HLA mismatch S cells. All data are presented as mean ± standard deviation. *: $P \leq 0.05$; **: $P \leq 0.01$.

Contrarily, there are various reports which address the beneficial effects of cell culture in media supplemented with HP or PL and suggest them as great FBS substitutes (23-25). For example, it was shown that PL as a substitute for FBS could enhance the transdifferentiation process of adipose-derived cells into cardiac linages (26).

Proliferation and cytogenetic assessment determined the preserved characteristic of cultured cells in medium supplemented with HS. Flowcytometry analysis showed that isolated and cultured CDCs in both FBS and HS groups were positive for mesenchymal and cardiac surface markers while negative for endothelial and hematopoietic lineage markers although the percentage of positive cells for each marker was partially different between these two groups. In agreement, there are various reports pointing that distinct cell types which were cultured in media supplemented with HS had more proliferation and differentiation potential compared to FBS-cultured cells. On the other hand, HS could preserve the typical characteristics of cultured cells the same or even better than FBS (21, 27-29). In contrast, a previous study exhibited the reduced growth rate and altered characteristics of CDCs in HS and introduced B27 as the most efficient FBS replacement (30). Worth noting, cell culture with B27 would be more expensive than HS and might not be affordable for patients. Furthermore, they used the autologous HS which might affect differently from pooled allogeneic AB sera utilized in our study. However, in a recently published paper, it was demonstrated that mesenchymal stromal cells (MSCs) which were cultured in autologous HS had more proliferation and more resistance to external stress stimulus compared to cultured MSCs in FBS (31). It has been reported that cultured synovial MSCs in medium containing autologous HS can maintain and proliferate in complete HS as a transfer medium. The paper suggested the usage of 100% HS as an efficient preservation material for synovial MSCs before their transplantation (32). Moreover, batch to batch reproducibility of HS was examined and confirmed which makes it a suitable FBS replacement especially for clinical studies (33).

As patients with CVDs may suffer from blood pressure, obtaining autologous sera for culture applications may be challenging; therefore, establishment of human AB serum banks could advance the field of cardiovascular cell therapy; however, the immunological reactions of autologous or allogeneic CDCs after culture in HS was still challenging. We showed that CDCs cultured in medium supplemented with HS did not trigger the proliferation of lymphocytes. Similarly, in a study it was indicated that the transplantation of skeletal myoblast cultured in autologous human serum could enhance the patients' heart performance without any malignant cardiac arrhythmia which already had been reported as the safety concern of myoblast transplantation. The authors associated these findings to the usage of autologous serum instead of FBS in cell culture medium which avoided the risk of microbial contamination and prevented the immunological and inflammatory adverse effects of cardiac cell therapy (34). Interestingly, the obtained data of our MLR assay exhibited the anti-proliferation effect of CDCs on stimulated lymphocytes. This observation was in line with previous studies which investigated the immuno-modulatory behavior of CSC (7). This phenomenon can be associated with different suggested mechanisms including the secretion of immuno-modulatory paracrine factors which is worth evaluating.

In the current study, we evaluated the effect of medium supplemented with FBS substitutes only in one concentration. As it is previously shown that the characteristics of cultured cells such as proliferation in media supplemented with HS is concentration-dependent (29, 35), studying the effect of lower and higher concentrations on CDCs' phenotype is recommended in the future studies. In addition, the evaluation of CDC differentiation propensity cultured in medium supplemented with HS is highly recommended for future work.

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Conflict of Interest Statement

The authors have no conflicting financial interest.

References

1. Shirzad N, Bordbar S, Goodarzi A, Mohammad M, Khosravani P, Sayahpour F, et al. Umbilical Cord Blood Platelet Lysate as Serum Substitute in Expansion of Human Mesenchymal Stem Cells. Cell J. 2017;19(3):403-14.

2. Gersh BJ, Sliwa K, Mayosi BM, Yusuf S. Novel therapeutic concepts: the epidemic of cardiovascular disease in the developing world: global implications. European heart journal. 2010;31(6):642-8.

3. Roth GA, Johnson C, Abajobir A, Abd-Allah F, Abera SF, Abyu G, et al. Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015. Journal of the American College of Cardiology. 2017;70(1):1-25.

4. Oh H, Ito H, Sano S. Challenges to success in heart failure: Cardiac cell therapies in patients with heart diseases. Journal of cardiology. 2016;68(5):361-7.

5. Chen C, Termglinchan V, Karakikes I. Concise Review: Mending a Broken Heart: The Evolution of Biological Therapeutics. Stem cells. 2017;35(5):1131-40.

6. Cho GS, Fernandez L, Kwon C. Regenerative medicine for the heart: perspectives on stem-cell therapy. Antioxidants & redox signaling. 2014;21(14):2018-31.

7. Sanz-Ruiz R, Fernandez-Aviles F. Autologous and allogeneic cardiac stem cell therapy for cardiovascular diseases. Pharmacological research. 2018;127:92-100.

8. Bianconi V, Sahebkar A, Kovanen P, Bagaglia F, Ricciuti B, Calabro P, et al. Endothelial and cardiac progenitor cells for cardiovascular repair: A controversial paradigm in cell therapy. Pharmacology & therapeutics. 2018;181:156-68.

9. Vahdat S, Mousavi SA, Omrani G, Gholampour M, Sotoodehnejadnematalahi F, Ghazizadeh Z, et al. Cellular and molecular characterization of human cardiac stem cells reveals key features essential for their function and safety. Stem cells and development. 2015;24(12):1390-404.

10. Li TS, Cheng K, Lee ST, Matsushita S, Davis D, Malliaras K, et al. Cardiospheres recapitulate a niche-like microenvironment rich in stemness and cell-matrix interactions, rationalizing their enhanced functional potency for myocardial repair. Stem cells. 2010;28(11):2088-98.

11. Davis DR, Kizana E, Terrovitis J, Barth AS, Zhang Y, Smith RR, et al. Isolation and expansion of functionally-competent cardiac progenitor cells directly from heart biopsies. Journal of molecular and cellular cardiology. 2010;49(2):312-21.

12. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, et al. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circulation research. 2004;95(9):911-21.

13. Usta SN, Scharer CD, Xu J, Frey TK, Nash RJ. Chemically defined serum-free and xeno-free media for multiple cell lineages. Annals of translational medicine. 2014;2(10):97.

14. Panchalingam KM, Jung S, Rosenberg L, Behie LA. Bioprocessing strategies for the large-scale production of human mesenchymal stem cells: a review. Stem cell research & therapy. 2015;6:225.

15. Tekkatte C, Gunasingh GP, Cherian KM, Sankaranarayanan K. "Humanized" stem cell culture techniques: the animal serum controversy. Stem cells international. 2011;2011:504723.

16. Karnieli O, Friedner OM, Allickson JG, Zhang N, Jung S, Fiorentini D, et al. A consensus introduction to serum replacements and serum-free media for cellular therapies. Cytotherapy. 2017;19(2):155-69.

17. Angelini F, Ionta V, Rossi F, Miraldi F, Messina E, Giacomello A. Foetal bovine serum-derived exosomes affect yield and phenotype of human cardiac progenitor cell culture. BioImpacts : BI. 2016;6(1):15-24.

18. Monsanto MM, White KS, Kim T, Wang BJ, Fisher K, Ilves K, et al. Concurrent Isolation of 3 Distinct Cardiac Stem Cell Populations From a Single Human Heart Biopsy. Circulation research. 2017;121(2):113-24.

19. Gaetani R, Feyen DA, Doevendans PA, Gremmels H, Forte E, Fledderus JO, et al. Different types of cultured human adult cardiac progenitor cells have a high degree of transcriptome similarity. Journal of cellular and molecular medicine. 2014;18(11):2147-51.

20. Poloni A, Maurizi G, Serrani F, Mancini S, Discepoli G, Tranquilli AL, et al. Human AB serum for generation of mesenchymal stem cells from human chorionic villi: comparison with other source and other media including platelet lysate. Cell proliferation. 2012;45(1):66-75.

21. Tunaitis V, Borutinskaite V, Navakauskiene R, Treigyte G, Unguryte A, Aldonyte R, et al. Effects of different sera on adipose tissue-derived mesenchymal stromal cells. Journal of tissue engineering and regenerative medicine. 2011;5(9):733-46.

22. Kocaoemer A, Kern S, Kluter H, Bieback K. Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue. Stem cells. 2007;25(5):1270-8.

23. Ben Azouna N, Jenhani F, Regaya Z, Berraeis L, Ben Othman T, Ducrocq E, et al. Phenotypical and functional characteristics of mesenchymal stem cells from bone marrow: comparison of culture using different media supplemented with human platelet lysate or fetal bovine serum. Stem cell research & therapy. 2012;3(1):6.

24. Perez-Ilzarbe M, Diez-Campelo M, Aranda P, Tabera S, Lopez T, del Canizo C, et al. Comparison of ex vivo expansion culture conditions of mesenchymal stem cells for human cell therapy. Transfusion. 2009;49(9):1901-10.

25. Juhl M, Tratwal J, Follin B, Sondergaard RH, Kirchhoff M, Ekblond A, et al. Comparison of clinical grade human platelet lysates for cultivation of mesenchymal stromal cells from bone marrow and

adipose tissue. Scandinavian journal of clinical and laboratory investigation. 2016;76(2):93-104.

26. Siciliano C, Chimenti I, Bordin A, Ponti D, Iudicone P, Peruzzi M, et al. The potential of GMP-compliant platelet lysate to induce a permissive state for cardiovascular transdifferentiation in human mediastinal adipose tissue-derived mesenchymal stem cells. BioMed research international. 2015;2015:162439.

27. Yoon AY, Yun S, Yang H, Lim YH, Kim H. Expression of tight junction molecule in the human serum-induced aggregation of human abdominal adipose-derived stem cells in vitro. Development & reproduction. 2014;18(4):213-24.

28. Tateishi K, Ando W, Higuchi C, Hart DA, Hashimoto J, Nakata K, et al. Comparison of human serum with fetal bovine serum for expansion and differentiation of human synovial MSC: potential feasibility for clinical applications. Cell transplantation. 2008;17(5):549-57.

29. Lindroos B, Aho KL, Kuokkanen H, Raty S, Huhtala H, Lemponen R, et al. Differential gene expression in adipose stem cells cultured in allogeneic human serum versus fetal bovine serum. Tissue engineering Part A. 2010;16(7):2281-94.

30. Chimenti I, Gaetani R, Forte E, Angelini F, De Falco E, Zoccai GB, et al. Serum and supplement optimization for EU GMP-compliance in cardiospheres cell culture. Journal of cellular and molecular medicine. 2014;18(4):624-34.

31. Nazari-Shafti TZ, Xu Z, Bader AM, Henke G, Klose K, Falk V, et al. Mesenchymal Stromal Cells Cultured in Serum from Heart Failure Patients Are More Resistant to Simulated Chronic and Acute Stress. Stem cells international. 2018;2018:5832460.

32. Mizuno M, Katano H, Otabe K, Komori K, Kohno Y, Fujii S, et al. Complete human serum maintains viability and chondrogenic potential of human synovial stem cells: suitable conditions for transplantation. Stem cell research & therapy. 2017;8(1):144.

33. Dos Santos VT, Mizukami A, Orellana MD, Caruso SR, da Silva FB, Traina F, et al. Characterization of Human AB Serum for Mesenchymal Stromal Cell Expansion. Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie. 2017;44(1):11-21.

34. Chachques JC, Herreros J, Trainini J, Juffe A, Rendal E, Prosper F, et al. Autologous human serum for cell culture avoids the implantation of cardioverter-defibrillators in cellular cardiomyoplasty. International journal of cardiology. 2004;95 Suppl 1:S29-33.

35. Ferro F, Spelat R, Beltrami AP, Cesselli D, Curcio F. Isolation and characterization of human dental pulp derived stem cells by using media containing low human serum percentage as clinical grade substitutes for bovine serum. PloS one. 2012;7(11):e48945.