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FULL LENGTH PAPER

Human umbilical cord serum as an alternative to fetal bovine serum for in vitro expansion of umbilical cord mesenchymal stromal cells

Elaheh Afzal · Mohammad Pakzad · Masoumeh Nouri · Reza Moghadasali · Morteza Zarrabi

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Abstract In the use of bovine fetal serum (FBS) there is concern about the possibility of disease transmission from animal to human. Therefore, it seems necessary to create culture conditions free of animal serum, especially in cell therapy. The aim of this study was to evaluate the feasibility of replacing human umbilical cord serum (hUCS) with FBS for in vitro expansion of umbilical cord mesenchymal stromal/stem cells (UC-MSCs). Here, UC-MSCs were cultured for five days in media supplemented either by hUCS or commercial FBS (Gibco and HyClone) to compare their viability, proliferation, morphology, Immunophenotype and differentiation potential. Our data shows that use of 5% and/or 10% hUCS, resulted in a tenfold increase in the number of MSCs; While in the presence of commercial FBS, this figure reached a maximum of five times. Notably, the rate of cell proliferation in the group containing 2% hUCS was the same as the groups containing 10% commercial FBS. Furthermore, there was no significant difference between groups in terms of viability, surface markers, and multilineage differentiation potential. These results demonstrated that hUCS can efficiently replace FBS for the routine culture of MSCs and can be used ideally in manufacturing process of UC-MSCs in cell therapy industry.

Keywords Fetal bovine serum · Human umbilical cord blood serum · Human umbilical cord-derived mesenchymal stem/stromal cells

E. Afzal · M. Nouri · M. Zarrabi (✉)
Research and Development Department, Royan Stem Cell Technology Company, Cord Blood Bank, Tehran, Iran
e-mail: m.zarrabi@rsct.ir

E. Afzal
e-mail: e.afzal@rsct.ir

M. Nouri
e-mail: m.nouri@rsct.ir

M. Pakzad · R. Moghadasali
Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
e-mail: pakzad_m@ymail.com

R. Moghadasali
e-mail: rezamoghadasali@yahoo.com

M. Zarrabi
Department of Regenerative Medicine, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran 19395-4644, Iran

Introduction

Mesenchymal stem cells (MSCs), also known as mesenchymal stromal cells, are novel cell-based biologic therapeutic which in recent years, have been widely used for cell therapy applications (Fibbe et al. 2003). The main issue with using MSCs is their extremely low frequency, with one mesenchymal cell for every 10,000 to 20,000 cells in the bone marrow (Gronthos et al. 1994). As a result in vitro expansion of MSCs

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is a prerequisite for research as well as cell therapy purposes. The most common widely used type of supplementation to provide the essential growth factors needed for cell survival, adhesion, and growth is fetal bovine serum (FBS), which is derived from the bovine fetus. However, for therapeutic purposes, avoiding the cross-contamination of animal pathogens to hMSCs through the replacement of FBS with the serums derived from human tissues should be considered.

Transplantation of mesenchymal cells cultured in the presence of animal serum can causes some complications. First, the immune system of the recipient recognizes animal tissue proteins as xenogenic antigens, resulting in immunological inflammatory reactions and, eventually, transplant rejection (Spees et al. 2004). In addition, despite the high sterile conditions, the risk of transmission of bacterial and viral infections, especially prions, has not been eliminated. In the latter case, the patient is at risk for Creutzfeldt-Jacob disease (bovine spongiform encephalopathy) (Dedrick 1997, Klein et al. 1993, Will et al. 1996). Animal serum residues (approximately 7 to 30 mg) in the cytoplasm of cultured mesenchymal cells exacerbate these issues and highlight the need for a suitable replacement (Muller et al. 2006).

Given the aforementioned ethical and bio-safety concerns about the use of animal serum, so far the idea of using human-derived serum has been considered by many researchers (Schallmoser et al. 2008). However, there are controversial results about the efficacy of human serum, reinforcing the need for more research. While some studies have found that autologous serum outperforms FBS, others have found that FBS surpass the autologous serum (Anselme et al. 2002; Koller et al. 1998; Kuznetsov et al. 2000; Shahdadfar et al. 2005). Furthermore, the results of some studies indicate that the effect of FBS and human serum on the growth of mesenchymal stem cells is the same (Doucet et al. 2005; Muller et al. 2006; Yamamoto et al. 2003).

The results of recent studies show that human umbilical cord serum (hUCS) which is a reliable source of many growth factors and biologically active molecules, in addition to removing barriers to the use of FBS, have the ability to optimize culture conditions for cell therapy purposes and allows the production of mesenchymal cells on a large scale (Bernardo et al. 2007; Reinisch et al. 2007). Importantly, it is xeno-free and has remarkably lower immunogenicity. Furthermore, UCS appears to be a suitable candidate for FBS replacement because of the non-invasive and simple production method, as well as the low risk of bacterial/viral contamination. However, further research into the use of UCS as a supplement for routine cell culture is required to establish cord serum for medicinal application.

Due to the progressive research for the therapeutic application of allogenic MSCs derived from umbilical cord, we decided to evaluate the effects of hUCS, as a culture medium supplement on the (i) morphology and proliferation of UC-MSCs, (Baradaran-Rafii et al. 2018) the immunophenotype characterization, and (iii) the multipotent characteristics of UC-MSCs in parallel with common FBS supplements (GIBCO and HyClone).

Materials and methods

Serum preparation

Two types of serum were used in the present study, human umbilical cord-derived serum (hUCS) and commercial fetal bovine serums. The umbilical cord serums included in the present study were obtained from the cord blood samples of nine consenting mothers who had no medical history of serious infectious diseases. The blood samples were collected without anti-coagulants and allowed to clot at room temperature and then centrifuged at 450 g for 10 min at 20 °C. The hUCS was filtered through the 0.22 µm pore size. Then, three serum samples were pooled in each batch and tested for HBV, HIV, CMV, HCV and HTLV 1,2 viral contaminations. Microbiological analysis was included aerobic and anaerobic tests, mycoplasma and endotoxin assays. HyClone and GIBCO FBS were purchased from Thermo Fisher and life technology, respectively.

Culture of UC-MSCs

Umbilical cord mesenchymal stem cells (at passage number 4) were donated by the stem cell bank of Royan Stem Cell Technology Company. The UC-MSCs were initially isolated and cultured in FBS-containing medium. Briefly, upon receiving the frozen stock, the cells were thawed and cultured using

Iscove's modified Dulbecco's medium (IMDM; Thermo Scientific), 1% L-Glutamin (Invitrogen, Carlsbad, CA, USA), 1% penicillin–streptomycin (Invitrogen), and 10% FBS in T25 culture flask at 37◦C in 5% CO2 incubator. At 90% confluency, the cells were dissociated with trypsin/EDTA (Invitrogen) and 10^5 cells/ one well of a 6-well dish were culture in different groups (1) serum free medium (Negative Control), (2) 10% FBS (HyClone), (3) 10% FBS (Gibco), (4) 2% hUCS, (5) 5% hUCS, (6) 10% hUCS.

Proliferation and viability of UC-MSCs

UC-MSCs were dissociated and counted by trypan blue, 5 days after replating. Fold expansion was determined by dividing the total number of viable cells after 5 days of culture, by the number of viable cells in the first day of culture.

Immunostaining for MSC markers

Phenotypic analysis of expanded UC-MSCs was performed using flow cytometery. Cells were blocked with PBS-BSA 1% and were stained with appropriate amount of fluorochrome-conjugated antibodies, CD45FITC/CD34PE (BD Biosciences, 341,071), CD90FITC (BD Biosciences, 555,595), and CD73PE (BD Biosciences, 550,257), CD105PE (BD Biosciences, 560,839) Following incubation at room temperature for 20 min. The negative population was determined using relevant isotype control antibodies. At least 10^4 events were acquired on a Partec PAS system (Germany), and data were analyzed with FloMax software. Data (Mean ± standard deviation) were collected from three independent experiments.

Differentiation potential

Expanded UC-MSCs in HyClone, Gibco, and 5% UCS groups were cultured for 21 days according to the suggested protocol of Thermofisher differentiation kits (A1007101; StemPro® Osteogenesis Differentiation Kit; A1007201 StemPro® Adipogenesis Differentiation Kit; A1007001; StemPro® Chondrogenesis Differentiation Kit). Oil red O (Sigma-Aldrich, O0625) and Alizarin Red S (Sigma-Aldrich, A5533) demonstrated adipogenesis and osteogenesis, respectively. Chondrogenic differentiated cells were processed as paraffin-embedded blocks and were stained with Alcian blue (Sigma-Aldrich, A3157).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 6.00 for windows. All the experiments were performed in triplicates and the results were presented as Mean ± SD. Dunnett's multiple comparisons test against control group (10% HyClone FBS) were performed by one-way ANOVA supplemented with Tukey's HSD post-hoc test. Differences were considered statistically significant at $P \leq 0.05$.

Results

Evaluation of morphology, proliferation and survival of UC-MSCs

As shown in Fig. 1A, there is no notable difference in the morphology of UC-MSCs cultured in medium containing either FBSs or UCS. Expanded cells in all groups had typical fibroblast-like appearance of MSCs. The only significant difference was the cellular density in 5 and 10% concentration of UCS compared to the other groups.

According to our results, 5 days after culture, the number of MSCs in FBS groups is similar to the 2% UCS (Fig. 1B). However, there is a significant increase in the number of MSCs groups containing 5 and 10% of UCS. It is noteworthy that compared to the first day, the number of cells in the presence of Gibco and HyClone FBS increases fivefold, while it increases tenfold in 5%- and 10%- UCS groups. Furthermore, the result of trypan blue assay for cell viability is demonstrated in Fig. 1C. Based on our data, there was no significant difference in survival between the different experimental groups (n = 9, mean ± SD, *** ≤ 0.005, ****0.0005).

Evaluation of phenotype identity of UC-MSCs

In terms of expression of specific markers of mesenchymal stem cells including CD90, CD73 and CD105 indices were examined, but no significant difference was observed between different groups. The expression of differentiating markers of CD34 and CD45

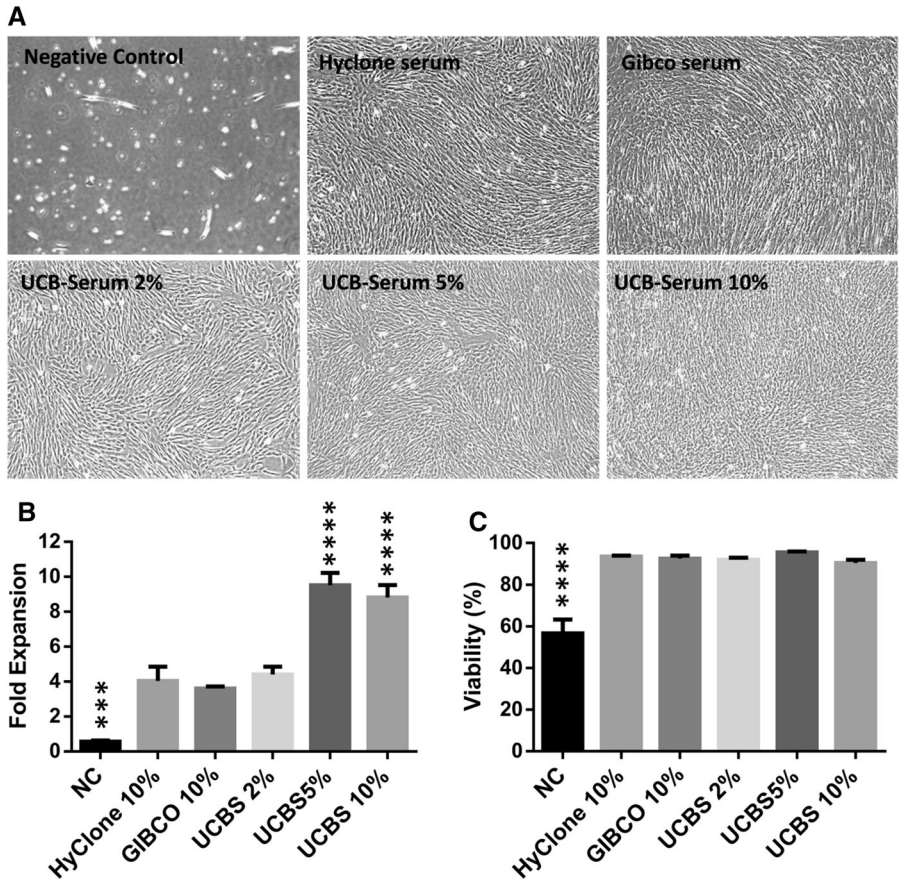

Fig. 1 **A** Phase-contrast microscopy of hUCB-MSCs cultured either in serum-free medium or 10% FBS (HyClone, Gibco) and different concentration of UCBS (2%, 5%, 10%) for 5 days, at 10X magnification. **B** Fold expansion of hUCB-MSCs cultured under different media supplementations for 5 days. Fold expansion was determined by dividing the total number of viable cells after 5 days of culture, by the number of viable cells in the first day of culture. **C** Viability of hUCB-MSCs cultured under different media supplementations for 5 days. n = 3 (mean ± SD), statistically significant difference compared with the HyClone group, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$

hematopoietic cells was also very low and less than half a percent in all groups (Fig. 2).

Multilineage differentiation of UC-MSCs

Microscopic observation of stained cells showed that proliferated mesenchymal stem cells in the presence of umbilical cord serum could differentiate into adipose and bone cells as well as chondrocytes (Fig. 3).

Discussion

The application of cellular therapies is growing enormously in a wide range of medical fields, often using hMSCs. Despite this, the availability of hMSCs limits their clinical applications due to the small number of cells that can be safely isolated per unit tissue volume. As a result, hMSCs must be extended and differentiated ex vivo before being used as a therapeutic, hence the need for in vitro culture. MSCs are valuable therapeutic candidates isolated from various sources that are being extensively used in various clinical trials. Effective therapeutic use of these cells is substantially connected with in vitro growth. To achieve this goal, high-quality and clinically relevant supplies of enriched growth media are required to provide the essential nutrients necessary for cell survival, adhesion, and growth. In this regard, the replacement of bovine fetal serum with human cord blood serum is currently receiving a lot of attention.

In this study, we attempted to improve the culture conditions for MSCs using human serum.

The human serum used in the present study was the human cord blood-derived serum, that has previously been demonstrated to have beneficial effects on the isolation, proliferation, and differentiation of human amniotic fluid stem cells (hAFS) and Wharton's jelly mesenchymal stromal cells (Julavijitphong et al. 2014; Phermthai et al. 2016).

The aim of this study was to examine the feasibility of using hUCS for culturing MSCs. Our results

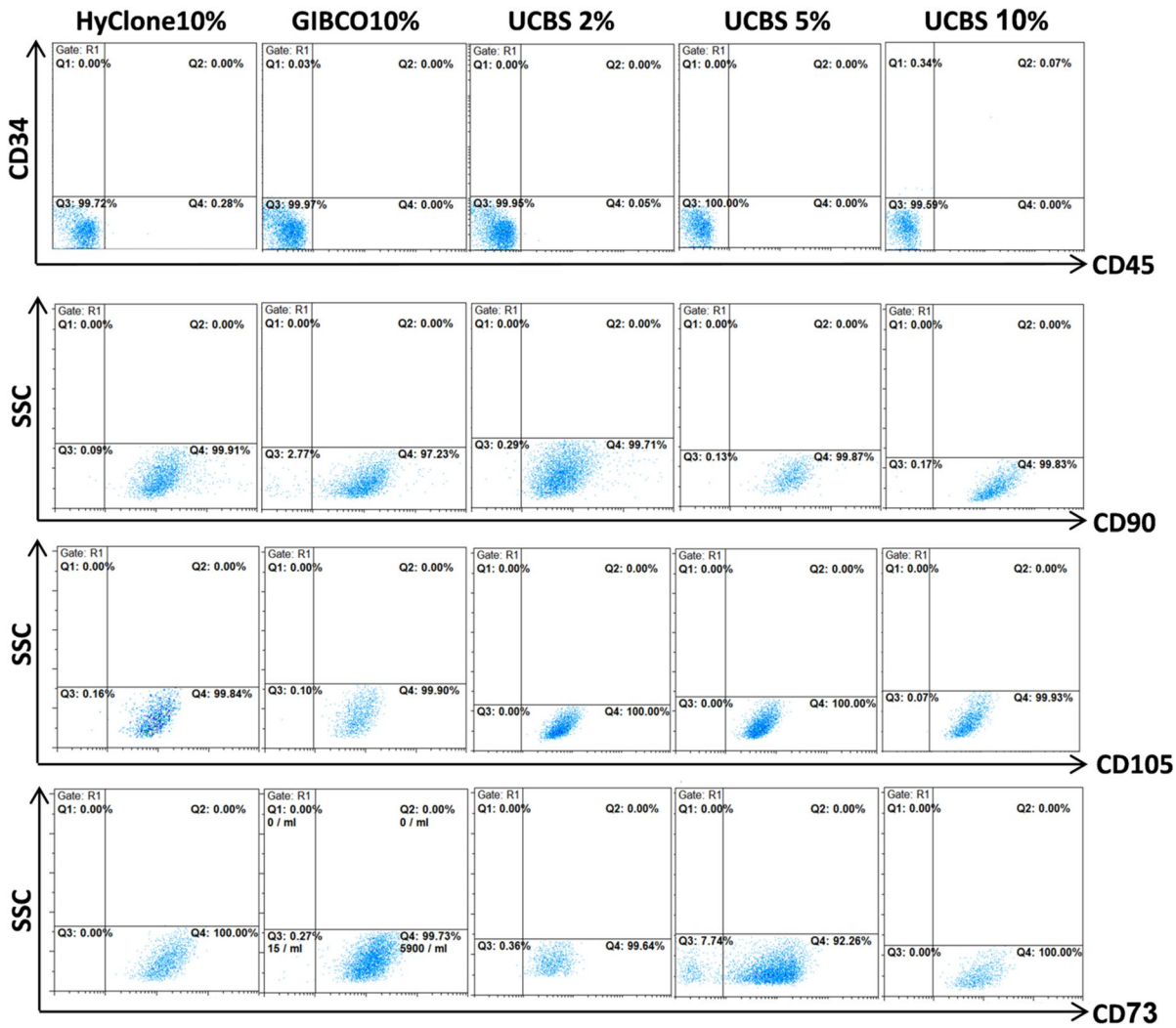

Fig. 2 Representative flow cytometrey histograms of the surface marker expression of UCB-MSCs cultured either in FBS (Hyclone and GIBCO) or UCBS. As illustrated, cells from all cultures expressed high levels of CD90, CD105, CD73 but not CD34/CD45 on their surface

showed that at equal and even lower concentrations of cord blood serum compared to commercial animal serums, the rate of cell proliferation was higher. Because 5 days after culturing umbilical cord tissue-derived mesenchymal stem cells, the total number of cultured cells in the groups containing 5% and 10% umbilical cord blood serum increases by about 10 times. However, in the presence of GIBCO and HyClone commercial serums, this figure reaches a maximum of 5 times the initial number. It should be noted that the rate of cell proliferation in the group containing 2% of cord blood serum is the same as the group of GIBCO and 10% of HyClone. On the other hand, there was no significant difference in survival rates between different experimental groups. We further tested the expression of specific mesenchymal surface markers including CD105, CD90 and CD73, after 5 days culture in the presence of hUCS. The results demonstrated that UCS-expanded MSCs maintained their immunophenotype with normal morphology. In addition, these MSCs have been shown to differentiate into bone, cartilage, and adipose tissues, confirming the multipotency of these cells. Therefore, the results of this study are in line with studies on the

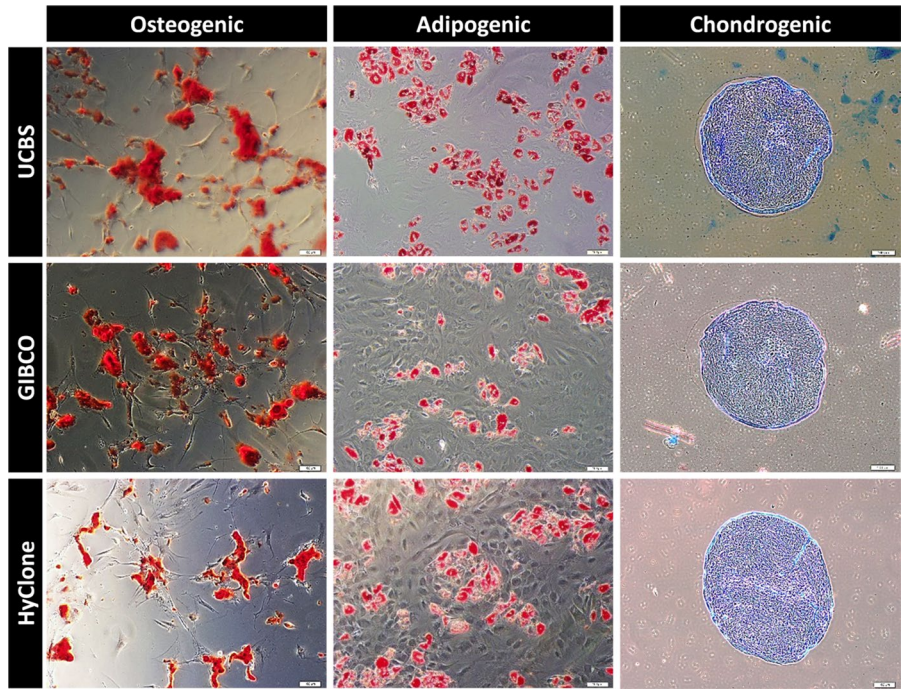

Fig. 3 Osteogenic, adipogenic and chondrogenic differentiation of UCB-MSCs in the presence of UCBS 5% compared with HyClone 10% and Gibco 10%

replacement of cord blood serum with animal serum for research and therapeutic purposes.

According to several studies in the field of umbilical cord blood proteomics, it has been found that human cord blood serum contains growth factors, cytokines and immune-mediated factors that affect the proliferation and function of stem cells. Concentrations of cytokines and growth factors in human umbilical cord blood serum have been measured by various analyzes. Compared with peripheral blood serum, cord blood serum significantly contains higher concentrations of molecules including interleukins 4, 5, 6, 7, 10 and 15 and MCP-1, SCF and SDF, as well as growth factors involved in regenerative processes such as EGF, TGF-α, TGF-β, FGF, PDGF, VEGF, NGF (Huang et al. 2019).

In conclusion, based on our result, FBS and hUCS present relevant similarities. These observations suggest that hUCS could be considered as an alternative to FBS for in vitro production of MSCs for cell-based therapies and for clinical applications. Nevertheless, investigation must deepen regarding the applicability of hUCS through the entire hMSCs production process, and studies are ongoing as for their suitability for the primary isolation of cells from source tissues, as well as for cryopreservation and final formulations of hMSCs derived therapeutic products. Notably; the important issues of using serum are pooling the serum from different donors and the lot-to-lot variability of hUCS. To control the quality of hUCS, several issues need to be considered such as the donors must not have any history of infectious diseases, the serum producing processes has to be carried following a strictly aseptic technique, or the serum has to be tested against contamination of endotoxins. Importantly, the ability of each batch of serum should be preliminary tested for culturing the MSCs prior to use the serum in a large scale.

Conclusion

In conclusion, these results demonstrated that hUCS is an effective alternative source of serum for supplementation in the culture medium of MSCs. Not only does hUCS support the growth of MSCs, but also maintain the viability, immunophenotype as well as the differentiation potential of the UCS-expanded MSCs.These findings benefits the optimization of the xeno-free culture conditions of MSCs.

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Authors contributions Elaheh Afzal and Mohammad Pakzad performed the experiments. Elaheh Afzal, Masoumeh Nouri and Reza Moghadasali wrote the manuscript. Masoumeh Nouri, Reza Moghadasali and Morteza Zarrabi contributed to the concept and design. All authors read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article and in supplementary figures.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All the experiments in this study were reviewed and approved by the research ethics committee of Royan institute and was conducted in accordance with the ethical principles and the national norms and standards for conducting medical research in Iran (IR.ACECR.ROYAN.REC.1400.163).

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