RESEARCH ARTICLE

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Effect of Replacement of Wharton Acellular Jelly With FBS on the Expression of Megakaryocyte Linear Markers in Hematopoietic Stem Cells CD34+

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Abstract

Objective: Animal environments for the growth of stem cells cause the transmission of some diseases and immune problems for the recipient. Accordingly, replacing these environments with healthy environments, at least with human resources, is essential. One of the media that can be used as an alternative to animal serums is Wharton acellular jelly (AWJ). Therefore, in this study, we intend to replace FBS with Wharton jelly and investigate its effect on the expression of megakaryocyte-related genes and markers in stem cells. **Materials and Methods:** In this study, cord blood-derived CD34 positive HSCs were cultured and expanded in the presence of cytokines including SCF, TPO, and FLT3-L. Then, the culture of expanded CD34 positive HSCs was performed in two groups: 1) IMDM culture medium containing 10% FBS and 100 ng / ml thrombopoietin cytokine 2) IMDM culture medium containing 10% AWJ, 100 ng / ml thrombopoietin cytokine. Finally, CD41 expressing cells were analyzed with the flow cytometry method. The genes related to megakaryocyte lineage including FLI1 and GATA2 were also evaluated using the RT-PCR technique. **Results:** The expression of CD41, a specific marker of megakaryocyte lineage in culture medium containing Wharton acellular jelly was increased compared to the FBS group. Additionally, the expression of GATA2 and FLI1 genes was significantly increased related to the control group. **Conclusion:** This study provided evidence of differentiation of CD34 positive hematopoietic stem cells from umbilical cord blood to megakaryocytes in a culture medium containing AWJ.

Keywords: Hematopoietic stem cells- CD34- Wharton Allular jelly- Megakaryocyte lineage

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Introduction

Hematopoietic stem cell transplantation (HSCs) is used to treat immunodeficiency diseases and hematological or non-hematological malignancies. Since about 100 million children are born worldwide each year, it can be concluded that the largest source of hematopoietic stem cells is cord blood. Also, the use of fetal umbilical cord blood compared to other sources has advantages such as a lower risk of graft-versus-host transplantation (GVHD), the number of primary umbilical cord blood progenitors is 10 times higher than that of bone marrow, and lower probability of the transmission of infectious agents particularly, cytomegalovirus (CMV) and Epstein Barr Virus (EBV), high colonization capacity and lack of need for high compatibility in terms of common leukocyte antigens (HLA) between the donor and the recipient. These factors have drawn researchers, attention to the use of fetal umbilical cord blood to produce stem cells (Gluckman, 2009; Zhong, 2010; Moghadasi, 2021).

However, in cord blood compared to other sources of stem cells, the number of HSPCs per unit of cord blood is low and each unit contains 2-4 × 10^6 cells per kilogram of body weight, and we use more to transplant hematopoietic stem cells. We need 2.5 X 10^7 hematopoietic stem cells per kilogram of body weight. So we are looking for a way to increase these hematopoietic stem cells, one of which is to culture these cells in vitro. The proliferation of human and animal cells in vitro requires factors such as specific culture media, specific cell culture conditions, and necessary cytokines that can be increased in the laboratory. The basic composition of these media is supplemented

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with animal serum, especially fetal bovine serum (FBS), which promotes cell growth and proliferation (Rauch, 2011; Emamgolizadeh Gurt Tapeh, 2020). The use of animal serums may increase the risk transmitting of diseases such as Mycoplasma and Prion, as well as the risk of zoonotic contamination by transmitting viruses such as bovine diarrhea virus, parvovirus, and Krotsfeld-Jakob virus (Muraglia, 2015; Chevallier, 2010; Tapeh, 2020) and also increase immune response against animal antigens (Russell, 2016 ; Tapeh, 2021). In addition, there are moral problems in using of bovine embryonic serums (Muraglia et al., 2015; Russell and Koch, 2016)

So we are looking to provide alternative environments that have a human resources and can prevent the risks of disease and other problems in bovine fetal serum. One of the media that can be used as an alternative to human-derived animal serums is Wharton acellular jelly (AWJ). Wharton's acellular jelly contains a wide range of growth factors. Growth factors seen in Wharton acellular jelly include platelet-derived growth factor (PDGF), epithelial growth factor (EGF), basal fibroblast growth factor (bFGF), acid fibroblast growth factor (aFGF), beta-growth metastatic factor 1 (TGF B) and insulin-like growth factor (IGF-I) (Sobolewski, 2005; Tapeh, 2021). There is a receptor for most of these molecules on hematopoietic stem cells and precursors of blood cells. All of these molecules affect cell proliferation and function and may alter proliferation relative to FBS (Astori et al., 2016). Due to the disadvantages of using animal serums in culture medium, healthy media with a human source such as Wharton acellular jelly can be used for hematopoietic stem cell transplantation (HSCT).

Materials and Methods

Sample Collection

Two cord blood samples through a heparin-soaked syringe (200 U / ml), sterile in 50 cc Falcon tubes, as well as 15 umbilical cord samples from both sides clamped in a sterile glass containing PBS from pregnant women referring to the gynecology and obstetrics ward of Al-Zahra Hospital in Tabriz, who had a cesarean delivery and a normal baby, were prepared randomly and with informed written consent. Umbilical cord blood samples were transferred to the laboratory in less than one hour to isolate CD34-positive hematopoietic stem cells, and cells were isolated from umbilical cord blood up to 8 hours after sampling. It should also be noted that cord and blood samples were stored at 4°C until cell separation.

Cell Culture

The isolated CD34 positive cells are replicated in IMDM medium containing 20% FBS. Cytokines including SCF100ng / ml, TPO 100ng / ml, and FLT3-L at a concentration of 100ng / ml were added to this medium for proliferating of cells. The cells proliferate in the above medium for 5 to 7 days at 37°C and 5% CO_2 and 95% humidity, then receive different factors and culture media for the next steps (megakaryocyte differentiation).

RNA extraction and cDNA synthesis

In this study, to extract the total RNA from the medium containing CD34-positive cells the RNX Plus solution kit was used according to the manufacturer's protocol. Subsequently, the extracted RNA was stored at -70°C. For cDNA synthesis using a high-efficiency kit (Bioneer, Alameda, CA, USA) a reaction mixture containing 500 ng of total RNA, 1 µL of reverse transcriptase) 200U/µ), 1 µl of RiboTM Lock RNAse Inhibitor (20U/µl), 2µl of 10 mM dNTP Mix, 2 µL of DNase/RNase free water, and 4 µl of 5X reaction buffer were used. . finally, the sample was slowly mixed and incubated at 37°C for 15 min and 85°C for 5 sec in a thermocycling machine for synthesizing cDNA.

Real Time PCR

The expression level of megakaryocyte-specific genes including FLi1 and GATA2 was quantitatively assessed by RT-PCR using SYBER green PCR master mix (amplicon 2x master mix) and the Corbett system. The reactions of RT-PCR contained 10 µL of 2x SYBR green mix, 1 µL of forward primer (10 pmol), 1 µL of reverse primer (10 pmol), 1 µL of cDNA template, and 7 µL of nuclease-free water in a total volume of 20 µL. The following cycling program was used on the qRT-PCR machine of Corbett: One cycle at 95°C for 15 min, 40–45 cycles, including 95°C for 20 sec, 60°C for 35 sec, 72°C for 25 sec and one final cycle for 5 min at 72°C. The expression ratio of GATA2 and Fli1 mRNA was evaluated through the $2^{(-\Delta\Delta CT)}$ method. We used the beta-actin gene as an internal control. Additionally, we analyzed the melting curve for evaluating the quality of RT-PCR using Rotor GeneTM 6000 Real Time Rotary Analyzer software. For each gene, a triplicate test was performed.

How to prepare Wharton aCellular Jelly

Fifteen umbilical cord samples were obtained from Alzahra Hospital in Tabriz at various intervals under sterile conditions. The samples belonged to cesarean section infants who were referred to the laboratory after obtaining the informed consent of the mothers in an autoclaved glass containing 10 ml of 1% PBS / Pen / Sterep solution. Each cord was divided into 2 cm pieces. The umbilical cord parts are washed 3 times in PBS / Pen / Sterep1% solution, then in the next step, it is placed in 70% ethanol solution for 30 seconds. Then the ethanol is drained and the umbilical cord parts are washed 3 times with PBS / Pen / Sterep1% solution. The umbilical cord was then completely opened and Wharton jelly separated from the umbilical cord using a sterile scalpel. An average of 5 ml of Wharton jelly was isolated from each umbilical cord. The isolated Wharton jelly was added to a 50 ml sterile Falcon containing 25 ml of DMEM medium and incubated for 24 hours at refrigerator temperature and then centrifuged at 1400 rpm for 10 minutes. Excess cells and debris were separated on a falcon floor as a plate, and the supernatant was collected as Wharton's acellular gel and frozen at -80°C for use when needed.

Flow cytometry

The expression level of the specific marker of the

megakaryocytes, CD41, was assessed by flow cytometry, on days 0 and 3. Briefly, the cells were washed twice with PBS buffer containing 5% FBS and then incubated in the dark with the megakaryocyte-specific monoclonal antibody, CD41-FITC, at 25°C for 45 minutes. Finally, they were analyzed by FACS Calibur (Becton Dickinson, USA). Data was acquired by FACS Calibur equipped with the Cell Quest software package (BD Biosciences), and finally analyzed by flowing software (Turk University, Finland).

Statistical analyzing

All experiments were carried out in triplicate. The data obtained from the study were expressed as the mean ± SD and analyzed by Graph Pad Prism v8.2.4 (Graph Pad Software, San Diego, CA, USA). Statistical analysis for single comparisons was conducted by student's t-test, and P-values<0.05 were considered as statistically significant.

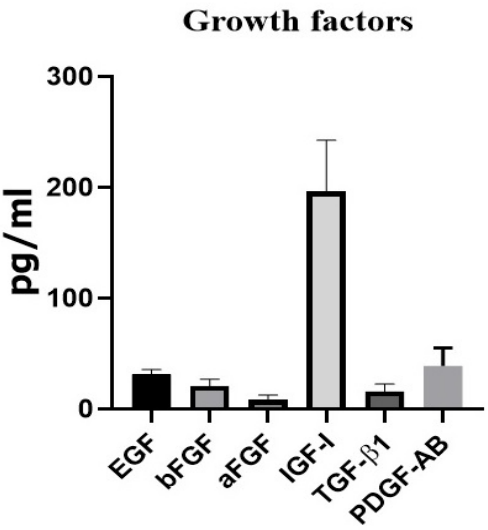

Figure 1. The Rate of Growth Factors of Wharton Acellular Jelly

Results

Results related to the rate of growth factors of Wharton ellular jelly

Growth factors in AWJ were measured by ELISA kit. The mean concentrations of Wharton's acellular growth factors are as follows:

E F 21.80 ± 4.63 pg / ml, bFGF 1.87 ± 0.97 pg / ml, aFGF 1.24 ± 0.81 pg / ml, PDGF-AB 26.44 ± 9.33 pg / ml, IGF-1 33.39 ± 13.82 pg / ml, TGF-β1 14.03 ± 6.58 pg / m.

Isolation of CD34 positive hematopoietic stem cells from Cord Blood

The mean number of umbilical cord blood mononuclear cells (MNCs) before isolation by the MACS method, the viability of MNCs, the average number of CD34 positive cells purified by the MACS method, and the percentage of CD34 positive cells purity are listed in Table 1.

Flow cytometry

Flow cytometric analysis was performed to evaluate the purity of CD34-positive hematopoietic stem cells isolated from the MACS column. The purity of these cells was 74%.

CD41 expressing cells on day 0

The percentage of CD41 expressing cells in IMDM medium containing FBS10% + TPO (100ng/ ml) as control group and IMDM medium containing AWJ10% + EPO (100ng/ ml) as test group were 7.69% and 20.18%,respectively,which was a significant difference between the control and test group.

CD41 expressing cells on day 3

The percentage of CD41 expressing cells in IMDM medium containing FBS10% + TPO (100ng/ ml) as a control group and IMDM medium containing AWJ10% + TPO (100ng/ ml) as test group were 27.09% and 32.39%, respectively, which was a significant difference between the control and test groups.

Table 1.

Sample Volume	Average number of MNCs	MNCs viability	Average number of CD34 positive cells	Purity of CD34 positive cells
100 ± 10	50 x 10^6	94%	1.85 x 10^6	74%

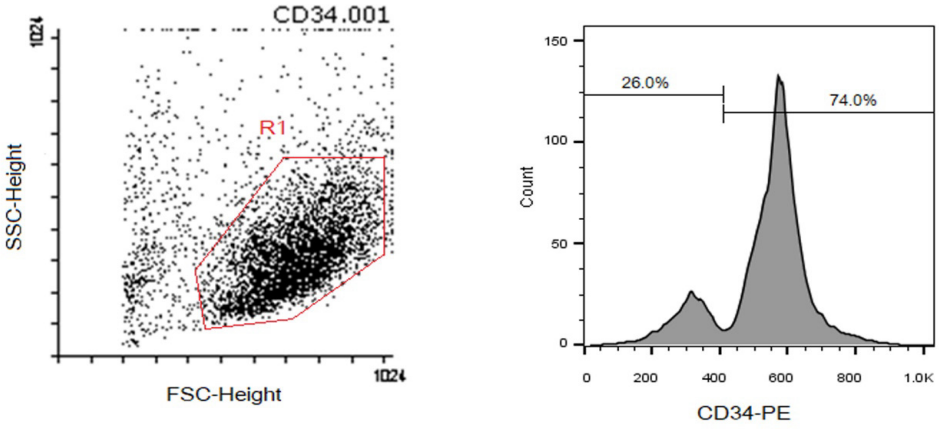

Figure 2. The Purity of CD34 Positive HSCs Isolated from the MACS Column

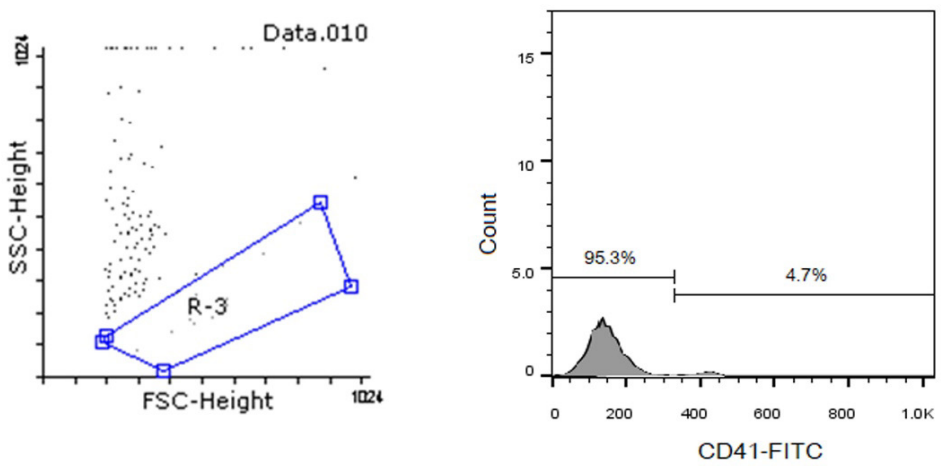

Figure 3. The Percentage of CD 41 Expressing Cells on Day 0

The expression of FLi1 and GATA2 genes on day 0 and day 3 in IMDM medium containing 10% FBS as control and IMDM medium containing 10% AWJ as test group.

The results of our study showed that the expression of the *GATA2* gene on days 0 and 3 in the medium containing Wharton jelly significantly increased compared to the control group. But changes in *Fli1* gene expression were not significant compared to the control group on day 0 and day 3.

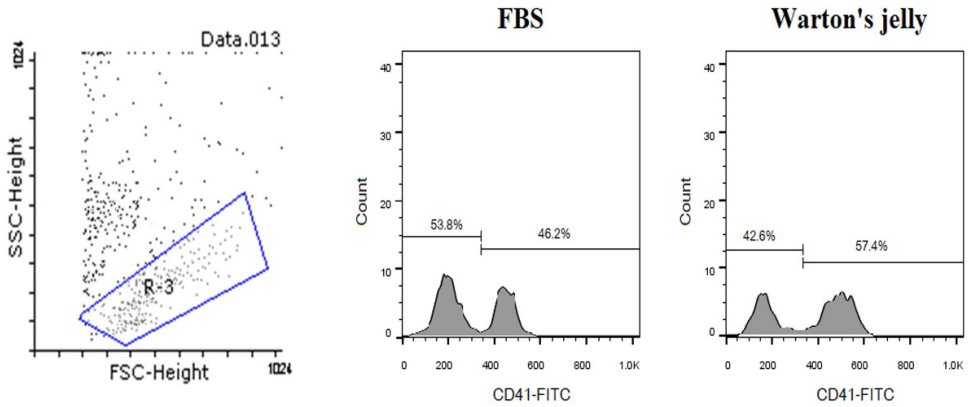

Figure 4. The Percentage of CD41 Expressing Cells on Day 3

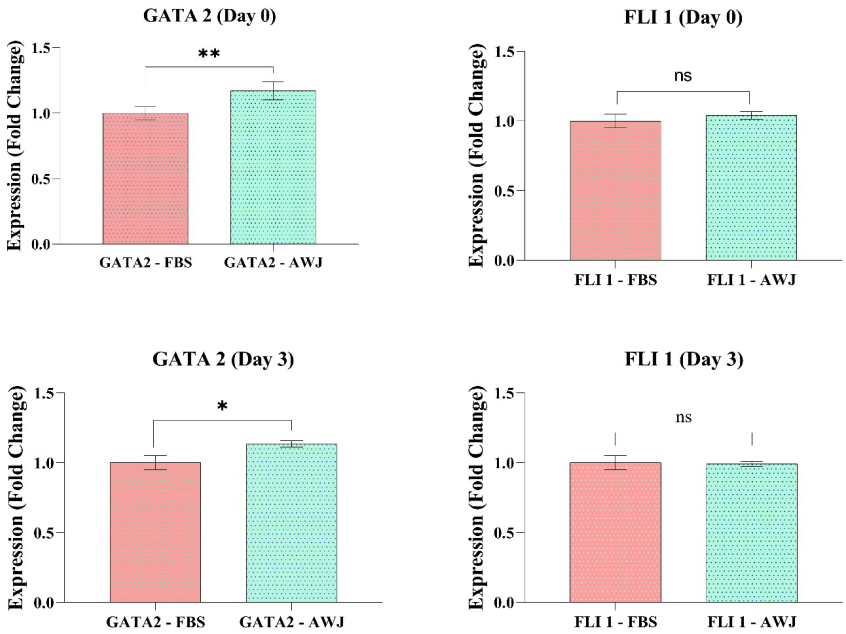

Figure 5. The Expression of Megakaryocyte Related Genes on day 0 and day 3

Discussion

Stem cell transplantation, including hematopoietic stem cells (HSCs), has achieved clinical therapeutic success in recent decades and is a viable and practical treatment for many cancers, malignant and non-malignant blood disorders, and immune system diseases (Zhao et al., 2019). There are several sources of HSCs, the most common of which are bone marrow (BM), G-CSF-stimulated peripheral blood, and umbilical cord blood (UCB). Using UCB as an alternative source for HSCs in transplantation has many advantages over other cellular sources, including early stem cell, higher colonization capacity, lower risk of GVHD, faster access, the need for less HLA compatibility between the donor and the transplant recipient, and the lower risk of transmitting infectious diseases, especially cytomegalovirus (CMV) (Gluckman, 2009; Zhong, 2010; Hashemzadeh, 2021). Clinical use of bovine fetal serum causes local inflammation, production of antibodies against foreign proteins, and transplant rejection due to heterogeneous immune response. Additionally, failure to remove unspecified toxic factors after spread in bovine fetal serum can cause damage (Horwitz et al., 2002; MacDermott and Bragdon, 1983; Phadnis et al., 2006). Since anaphylactic reports and Arthus-like immune reactions in patients after injection of grown lymphocytes in the environment containing bovine fetal serum and immunogenicity of cells killed in bovine fetal serum, there has been great concern about their use in therapeutic strategies (Selvaggi, 1997; Tapeh, 2021). The risk of transmission of prion and animal diseases associated with the use of bovine fetal serum is small, while the greater risk associated with the use of bovine fetal serum is the immunogenicity of non-native bovine fetal serum proteins in cultured cells (Shah, 1999; Spees et al., 2004).

Another problem with using FBS is human methods of collecting blood from embryonic calves. On average, about 106 embryonic calves are killed each year to collect about 105.5liters of bovine fetal serum (Hodgson, 1995; Jochems et al., 2002), which is completely contrary to the modern goals of laboratory biomedical research (Russell and Burch, 1959).

Accordingly, the need to replace animal serums with other sources of growth factors has attracted the attention of researchers in recent years. Wharton jelly contains a wide range of growth factors including platelet-derived growth factor (PDGF), epithelial growth factor (EGF), basal fibroblast growth factor (bFGF), fibroblast acid growth factor (aFGF), beta-1 growth transformant (TGF-1), and insulin-like growth factor (IGF-I) (Sobolewski et al., 2005). For most of these molecules, receptors are present on hematopoietic stem cells and precursors of blood cells. All of these molecules affect cell proliferation and function and may alter proliferation relative to FBS (Astori et al., 2016).

Wharton jelly is obtained from the umbilical cord, which is a biological waste, because it is from a human source, it does not have the mentioned disadvantages of using animal serums. Also, the availability and low cost of preparation make Wharton jelly a good candidate for replacement with animal serum.

To date, no study has been performed on the effect of replacement of Wharton acellular jelly with FBS on the pattern of proliferation and expression of markers related to blood cell lines, especially megakaryocyte cell lines in CD34 positive cells, so we evaluated the effect of Warton jelly replacement with FBS on the expression of markers related to the megakaryocyte lineage in CD34 positive cells.

In the present study, the percentage of CD41 expressing cells, which is a specific marker of megakaryocyte class, was significantly increased in a culture medium containing AWJ compared to the medium containing FBS. Additionally, the expression of the *GATA2* gene is significantly increased in media containing Wharton gel, it is inferred that Wharton gel differentiates stem cells into megakaryocytes. However, the expression of the Fli1 gene in a medium containing Wharton jelly does not change significantly compared to a medium containing FBS and AWJ. Based on this information, during differentiation, it is not necessary to increase gene expression in the following days and it is not expected that *GATA2* gene expression will increase on the third day compared to day 0. Because, due to changes in the expression of microRNAs, the expression of genes and markers is changed and differentiation into the megakaryocyte may be slightly reduced. Therefore, Wharton jelly differentiates stem cells to the megakaryocytes, but other factors may affect the expression of genes associated with megakaryocytes.

Previous studies also confirm the results of our study. We showed that the highest amount of growth factor was related to the IGF-1 factor and the lowest was PDGF. In conform with our study, Sobolewski et al., (2005) and Jadalannagari et al., (2016) reported that IGF-1 was the most abundant growth factor. IGF-1 and PDGF factors are involved in the proliferation and differentiation (Kehtari et al., 2019).

The results of a study by Jadalannagari et al., (2016) showed that the Wharton jelly-derived matrix is a biocompatible matrix that allows cell adhesion, penetration, growth, and proliferation of Wharton jelly-derived mesenchymal stem cells and bone marrow with acceptable mechanical properties in vitro and in vivo conditions (Staff, 2017).

In addition, a study by Beiki et al., (2017) Showed that scaffolds derived from Wharton acellular jelly can improve cell attachment, penetration, and proliferation. Additionally, this scaffold enhances wound healing without inflammatory responses (Beikiet al., 2017).

In conclusion, the results of this study revealed evidence of differentiation of umbilical cord blood-derived HSCs in the presence of 4.5IU / ml TPO and 20 ng / ml SCF cocktails in a culture medium containing Wharton's acellular jelly to the megakaryocyte lineage. Finally, our results showed that Wharton's acellular jelly is a suitable alternative to bovine fetal serum in a culture medium, but due to the presence of unknown substances in Wharton's acellular jelly, its use for hematopoietic stem cell transplantation requires more and more extensive research in this field.

Author Contribution Statement

All the authors made substantial contributions to the conception or design of the work;

Analysis, or interpretation of data for the work; AND

• Drafting the work or revising it critically for important intellectual content; AND

• Final approval of the version to be published; AND

• Agreement to be accountable for all aspects of the work in ensuring that questions related To the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Ethical Statement

This study was approved by Tabriz University of medical sciences

Conflict of interests

The authors declare no conflict of interests.

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