## Review Article

# Cell Identification, Characterization, and Documentation for Use in the Production of Biological Products

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## ABSTRACT

There is always a concern about the quality of cell-based products in terms of the contamination of the cells and their lack of efficiency. Therefore, it is of prime importance to ensure these cells' identity, purity, efficacy, and suitability for the production of biological products and diagnostic uses. Hence, cells must be identified, evaluated, documented, and stored to be used consistently and efficiently. With these conditions, vaccine manufacturers have a suitable reserve of efficient and valuable cells for the production and quality control of biological products. In this review article, a strategic plan was drawn for cell-substrate well-characterization and identification according to scientific principles, the author's work experience, and regulatory guidance for the optimal use of that cell in research and diagnostic studies especially for the biological product production process. For this purpose, all aspects of cell identification, cell evaluation, and cell characterization are discussed. Because of the importance of cell identity in the competence of a cell substrate, in the cell identification section, all aspects of cell identification, including general cell information and specific cell characteristics, especially in terms of cell passage history, cell storage conditions, and cell coding and labeling, were studied. In the part of cell evaluation and determination of cell characteristics, all required tests to determine cell characteristics from various aspects, including determination of cell identity, cell growth conditions, cell quality, efficiency, and the possibility of cell contamination with adventitious agents, including cellular, viral, bacterial, mycoplasma, and mycobacterial agents were introduced. Due to the importance of endogenous virus contamination, this topic is specially discussed. In addition, the stability of the cell both from the aspect of genetic stability and from the aspect of stability of cell efficiency, were discussed. In the end, while reviewing the necessary documents to be under the control of the cell for use in the laboratory, based on the studies conducted, the certificate of the cell has been compiled. Therefore, on this basis, the studied cell can be used for research and diagnostic studies of virology, especially for the production and quality control of biological products.

**Keywords:** Biological product, Cell culture, Characterization, Documentation

## 1. Context

Cell substrates are an integral part in producing the biological products. Nowadays, many vaccines, including important viral vaccines, are produced based on cell cultures or are subject to quality controls. One of the main regulations for achieving consistency and safety in the production of biological products is the characterization and testing of the starting material and the cell substrate. Since the use of cell cultures in the industry of biological products, there have been concerns regarding the use of cells in this industry due to the terrible events happened in this field (1). Therefore, establishing the requirements and regulations for using cell cultures in this industry was vital. The first requirements for cell substrates were published in 1959 by the World Health Organization (WHO) for the production of inactivated polio vaccine in primary cell cultures derived from all clinically healthy monkeys (2). These requirements were later revised in 1966 (3). After that, other primary cell cultures were used to produce other viral vaccines. In 1960, human diploid cells (HDCs) were developed and proposed as an alternative to primary cultures of monkey kidney cells for the production of poliovirus vaccine as well as other viral vaccines. The road to acceptance of diploid human cells was long and difficult. Primarily because some members of the scientific community believed that human diploid cells may contain a latent and unknown human oncogenic agent, and thus the possible risk factor for recipients of vaccines produced in human diploid cells was raised. Numerous discussions and conferences on the new data eventually led to the acceptance of human diploid cells as a substrate for the production of viral vaccines. The concept of Master Cell Bank (MCB) and Working Cell Bank (WCB) systems and the characterization of cell substrates were introduced during this period (4, 5). To create a high level of confidence, many preliminary tests for each vaccine series derived from human diploid cells, including tests of the cell substrate for adventitious agents, karyology, and tumorigenesis were performed (6, 7). In 1986, the WHO established a study group to investigate more deeply the cell substrate issues. The study group stated that there is no reason to reject continuous cell lines (CCLs) as substrates for the production of biological products and that they are generally acceptable because the production process eliminates viruses that are potentially pathogenic to humans and reduces DNA to an acceptable level or eliminates its biological activity (1). The study group's emphasis on infectious agents as the major risk factor was based in large part on experience in which virus and disease transmission had occurred through contaminated biological products (hepatitis B virus and AIDS virus (HIV) in factor eight). The WHO regulations and requirements on the use of CCLs for the production of biological products were published in 1987 (8). Therefore, it is crucial to select, characterize, document, and know the exact cell substrates to use in the production and quality control of biological products. Considering that in this review article, all aspects of cell study and recognition have been investigated, a specific strategy for cell investigation and evaluation will be provided to researchers. Therefore, in this study, a specific road map has been designed, which allows cell culture laboratories, as well as industries producing biological products based on cell culture, to identify, characterize, document, and bring under control the required cells based on this strategy, so that they can use the studied cell for specific processes continuously and especially on an industrial scale.

## 2. Evidence Acquisition

Considering the use of cell culture in the production and quality control of many biological products, it is critical to know the cell used, determine the characteristics of the cell, and storage conditions of the cell, compile the cell certificate and other related studies, because the requirement to produce a suitable product is to use a suitable and specific cell culture. Consequently, the process of cell identification and characterization should be cleared. This study aimed to introduce the steps and process of identification, characterization, and documentation of the cell that is used in the production of biological products. Some of the information presented in this article has been obtained through extensive searches in published articles in this field by the author, but most of these are the result of the author's summaries and the author's experiences in the field. For this purpose, the following steps should be taken:

1- Cell identification: One of the most important aspects of working with cells is compiling a complete certificate of the desired cell. For this purpose, complete information must be collected from the target cell. This information includes basic and general information about the cell. It should be noted that this information plays an effective role in cell identification, investigations, and evaluations (9).

2- Cell characterization: The second and basic part of the cell certificate is to perform the necessary tests to determine the cell characteristics and record the results. The results of the experiments can be used in making decisions and planning how to use the desired cell (10) (11).

3- Completing a cell documentation: Documenting all aspects of cell identification and evaluation ensures that the cell is under control and that safe storage of the cell is established. The most important document is the cell certificate. It is inevitable to have a cell certificate to

identify the cell in order to use that cell and check the characteristics and information of the cell. Additionally, the certificate of the cell is necessary for use in the documents of registration and acceptance of the products obtained from that cell by the relevant organizations. After collecting cell general information and conducting the necessary tests to identify and determine cell characteristics (cell characterization) and obtain the results of the tests performed, the cell certificate can be completed and formed. Furthermore, all the documents required to be under the control of the cell, including specifications, instructions, guidelines, protocols, forms, test records, and other necessary documents must be prepared, compiled, and controlled.

## 3. Results

To perform each of the mentioned steps to identify and determine the characteristics of the cells used in the production and quality control of biological products, and documentation of them, the following operations must be performed: (12-14):

1- Cell general requirements: In this part, all general and basic information about the cell is collected and analyzed. This information helps vaccine manufacturers and researchers to know the target cell and how to use that cell. This part of cell identification is divided into several groups of information as follows:

1-1- Cell general information: The general information of the cell indicates some basic and important information about the cell, which are specified as follows at this stage: In the general information section of the cell, the type of cell (e.g., primary cell types, diploid cells, cell lines, and stem cell) is specified, which is one of the basic information about the studied cell. Furthermore, the form of the cell during growth, which is one of the three states of sticky, semi-sticky, and floating, should be specified in this part as an attached cell or suspended cell. In this part, the tissue source of the desired cell and the host species of the tissue are specified, from which tissue and which organism the cell was obtained from. Other information in this section includes the source of the cell supplier, the primary code of the cell or the primary identification number of the cell from the supplier source, as well as the media used for the cell (the media used in the cell growth phase and also in the cell storage and maintenance phase).

1-2- Cell specifications: One of the most important cell specifications is cell passage information. At this stage, the first task is to determine the desired cell passage stage. From this point of view, the cells are placed in three stages: 1. Parent cell, 2. Master cell, and 3. Working cell (15, 16). The parent cell can be a cell purchased from an international or national supplier. The master cell is a cell that is prepared from the first cell passage of the parent cell, and the working cell is a cell that is prepared from the first cell passage of the master cell and is ready to be used for specific purposes. After this step, the passage information of the cells, including the passage number and date of the passage, must be determined. Considering that cell passage information is very important in cell efficiency, the information in this step will be very effective in using the cell (17). Other information that must be specified in this section is the number of stored cell cryo tubes and the number of cells in each cryo tube, which is obtained by counting cells and determining cell viability, which is explained in the following sections. It is worth mentioning that this cell passage information must be specified and recorded for each of the cell passage steps.

1-3- Cell storage conditions: In this section, all cell storage information for all three cell stages (Parent, master, and working cells) is recorded. This information includes the following: Storage date, nitrogen tank number, and can and canister number. This information should be specified separately for the main storage tanks of the cell as well as the backup storage tanks. Moreover, at this stage, the type of preservative used and the name of the operator performing the operation are recorded in the certificate (18).

1-4- Coding and labeling: To cell coding and labeling at each stage of the passage of the cell, it is necessary to act according to the internal rules and regulations of the group under activity. Although it does not matter if the coding and labeling instructions are based on valid references or internal rules, it is important that the cell identification code must cover the following minimum information so that the selected code has a specific identity:

- Cell name or cell name abbreviation
- Cell passage stage by mentioning all three stages or abbreviation of names
- The number of the cell passage
- Supplier of cell
- The year of preparation of the desired cell
- Batch number of cell preparation
- The number of cell cryo tubes compared to the total number of tubes prepared in that batch.

In addition, the labeling of the cell should also convey information about the cell so that an independent identity can be created for the cell label with the defined colors. The color intended for the label should be inclusive of the passage stage of the cell to minimize the possibility of possible errors between the passage stages. The designed code is engraved on the label, and with other specifications, they form the cell label (16).

2- Cell characterization: Cell characteristics are determined by performing tests in five groups of tests including the following (10, 19-21):

2-1- Identification tests: In this group of tests, two essential tests are performed:

2-1-1-Karyotyping: karyotype is the general appearance of the complete set of chromosomes in the cells. In this technique, mainly sizes, numbers, and shapes of the cells are specified. Karyotyping is the process by which a karyotype is discerned by determining the chromosome complement of a cell, including the number of chromosomes and any abnormalities. In karyotyping, pictures of the chromosomes from one cell are tacked, cut out, and arranged using size, banding pattern, and centromere positions as guides. Karyotype describes the amount of chromosome count and morphology of chromosomes under the light microscope. The derivation and study of karyotypes is part of cytogenetic studies. By performing cell karyology, the chromosomal map of the studied cell is determined (22).

2-1-2- Molecular tests: By designing the primer of the origin of the desired cell, the identity of the cell is investigated and evaluated by molecular method. Usually, based on the species of origin of the cell being studied, different primers are prepared from different animal species, which are usually the main species of origin of cells used in laboratories, such as human, cow, goat, pig, monkey, and so on. Next to the primer of the main species of origin of the studied cell, the PCR test is performed. Based on this, the origin of the desired cell is determined, and any possible cross-contamination of cells between species is also determined. (23, 24).

2-2- Cell growth profile: In this part, all tests specifying cell growth and cell growth conditions are performed as follows (25, 26):

2-2-1-Cell counting: With cell counting, the number of cells in the cryo tube is determined. Based on the determined number of cells, the next plan for using cell and cell passage is planned.

2-2-2- Viability test: Cell viability is a measure of the proportion of live, healthy cells within a population. Cell viability assays are used to determine the overall health of cells, optimize culture or experimental conditions, and measure cell survival following treatment with compounds, such as during a virus screen. There are several types of assays that can be used to determine the number of cell viability. These assays are based on various functions of cells, including enzyme activity, cell membrane permeability, cell adherence, adenosine triphosphate (ATP) production, co-enzyme production, and nucleotide uptake activity. Cell viability can also be assessed using cell toxicity assays that provide a readout on markers of cell death, such as a loss of membrane integrity (27).

2-2-3-Cell growth characteristics: Cells grow by cell division, which includes four major components: the G1, S, G2, and M phases in sequence. The cell growth curve is a graph that plots the number of cultured cells over time. By drawing the cell growth curve at different hours of logarithmic cell growth, the desired cell growth characteristics are evaluated (28).

2-2-4-Homogeneity: In this type of examination, the homogeneity and the way of cell growth in the desired system are examined and evaluated. The uniformity of the cell affects the efficiency of the cell as well as the durability of the cell (29).

2-3- Investigation of tumorigenic characteristics of cells: Cell systems to produce vaccines bear the risk of tumorigenicity or oncogenicity. Therefore, we should evaluate the tumorigenic and oncogenic potential of the cells following the requirements.

2-3-1- Tumorigenicity test: Tumorigenicity is defined as the property of a cell to form tumors when inoculated into an immunosuppressed animal model. The purpose of this test is to see if the desired cell is able to create a tumor after inoculation to laboratory animals. TPD50 (tumorigenic dose in 50% of animals) and the ability to form metastases are the characteristics of cell lines, which are considered to diagnose the tumor phenotype of a cell line. Line cell cultures obtained from rodents do not need to be tested for tumorigenicity, but human epithelial cells and all cell lines used to produce viral vaccines must be tested. Use an animal model that is known to be susceptible to tumor formation by tumorigenic cells. Use these as animal models because neonatal and aged immunocompromised rodents are relatively sensitive to reveal a tumorigenic phenotype. Thus, the most used animal for tumorigenesis tests is nude (nu/nu) (Athymic) mice because they have T cell defects (30, 31).

2-3-2- Oncogenicity test: Oncogenicity testing is required whenever test cells are tumorigenic in an immunosuppressed animal model. This study will check if the lysate of test cells or extracted DNA of the test cell can form tumors in the nude mice system. If a vaccine is manufactured in a cell substrate that has a tumorigenic phenotype, this cell line might carry a higher theoretical risk of containing oncogenes or oncogenic viruses (32).

2-4- Cell stability studies: In cell stability studies, which are an important evaluation of the cell, especially regarding the guarantee of cell efficiency, two types of studies are performed:

2-4-1- Study of genetic stability: The main concerns of the cell substrate stability are consistent production of the recombinant (rDNA) protein and retention of production capacity during storage under defined conditions. For this,

an analysis of the genetic stability of the production cells (MCB) and cells at the limit of *in vitro* cell age (End of Production Cells [EPC]) should be compared. The protein coding sequence for the rDNA protein of the expression construct needs to be verified. The DNA amplified by PCR from the sample DNA or cDNA prepared from RNA isolated from the test article (MCB and EPC) is used to perform the DNA sequencing analysis (5, 33).

2-4-2- Study of stability during storage: In these studies, the level of cell stability is determined, especially in terms of viability during the storage period of the cell. So that the cells are sampled at specific time intervals and cell viability is calculated. The stability and suitability of the cell substrate during cold storage must be proven. If records for storage show no deviation in characteristics, periodic testing to determine percent survival is not necessary. Each cell should be sampled once every five years to confirm suitability for production by calculating storage conditions.

2-5-Tests to determine the purity of the cell: In this group, the purity tests of the studied cell and the possible unwanted contamination in the cell are evaluated, including the following (34, 35):

2-5-1-Evaluation of bacteria and fungi in cells: This is determined by the culture of cells in microbiological media. This test is performed in a direct method using a thioglycollate culture medium for the growth of aerobic, microaerophilic, and anaerobic bacteria, and a TSB culture medium for the growth of bacteria and fungi.

2-5-2-Evaluation of adventitious viruses in cells: Detection of adventitious viruses in cell cultures, is done in the following ways:

- Cell extract culture in cells sensitive to viruses,
- Cell extract culture in the allantoic fluid of SPF embryonated eggs,
- Cell extract injection into the peritoneum of adult and infant mice,
- Molecular investigation for some specific viruses with specific primers.
- Detection of some viruses by transmission electron microscopy (TEM)

The most important bovine viruses as recommended, are Bovine viral diarrhea virus, Bluetongue virus, Bovine respiratory syncytial virus, Bovine adenovirus, Bovine parvovirus, Reovirus 3, and Rabies virus. Moreover, one of the most important viruses is retroviruses. Tests for retroviruses should include infectivity assays, reverse transcriptase (RT) assays, and transmission electron microscopy studies (36, 37).

2-5-3- Evaluation of mycoplasmas: The probability of cell contamination with mycoplasmas is determined by cell culture in specific environments. Mycoplasma detection is done by direct culture method and using PPLO agar and PPLO broth culture media. (38).

2-5-4- Detection of mycobacteria: To perform this test, the concentrated suspension extract prepared from the cell seed, which has been frozen and thawed in three stages, is cultured in tubes containing Lönstein's culture medium.

3- Completion of the cell certificate: In this way, by completing the above information in phases 1 and 2, a complete certificate of the studied cell is formed, and this certificate can be used and recorded for the cell. An example of this certificate is shown in tables 1 and 2.

4- Preparation of other cell documents: All the documents required for the control of the cell, which include the following, must be compiled:

4-1-Instructions and protocols: All necessary instructions, protocols, and guidelines for cell identification and characterization must be developed based on legal requirements (39).

4-2-Test Records: The necessary forms to record the information obtained from the tests to determine the characteristics of the cells under study should be designed, and all the test results should be recorded in the forms.

4-3-Specification: It is necessary to extract and record all the specifications of the tests necessary to determine the characteristics of the cell and apply them to different tests.

## 4. Conclusion

The use of cells in the biological industry goes back many years, so that for various reasons, today, the use of cells in these industries is inevitable for the production and quality control of these products. In addition, many cells are used in research and diagnostic studies (40).

Establishing a well-characterized cell is an important component of the quality control program for the manufacturing process of biological products (16, 41).

The first requirements for cell substrates were published in 1959 by the WHO for the production of inactivated polio vaccine in primary cell cultures derived from all clinically healthy monkeys (2). After that, other primary cell cultures were used to produce other viral vaccines.

Consequently, to use a cell in the production and quality control of biological products, the desired cell must be well investigated and identified, and complete information from this cell must be collected and controlled.

**Table 1:** An example of phase one of cell certificate (general cell information)

## Cell Name:

### 1. Cell General Requirements

#### *1.1. Cell General Information*

| Cell Type | | | | | Cell Source | | Cell Supplier | Cell Code | Medium Type | |
|---|---|---|---|---|---|---|---|---|---|---|
| Primary | Diploid | line | Attached | Suspended | Species | Tissue | | | Growth | Freeze |
| | | | | | | | | | | |

#### *1.2. Cell Specification*

| Cell | Passage Number | Passage Date | Number of tubes | Number of Cells/ tube |
|---|---|---|---|---|
| Parent Cell | | | | |
| Master Cell | | | | |
| Working Cell | | | | |

#### *1.3. Cell Storage Condition*

| Cell | Freeze Date | Main Storage | | | Backup Storage | | | Preservative |
|---|---|---|---|---|---|---|---|---|
| | | Nitrogen Tank Code | Canister Number | Cane Number | Nitrogen Tank Code | Canister Number | Cane Number | |
| Parent Cell | | | | | | | | |
| Master Cell | | | | | | | | |
| Working Cell | | | | | | | | |

#### *1.4. Cell Coding & Labeling*

| Cell | Code | Label Sample |
|---|---|---|
| Parent Cell | | |
| Master Cell | | |
| Working Cell | | |

| Comments: | |
|---|---|
| Prepared By: | Approved By: |

**Table 2:** An example of phase two of cell certificate (cell characterization)

## 2. Cell Characterization (*Master and Working Cell)*

| Tests | | | Applicability | Cell bank | Date | Operator | Result |
|---|---|---|---|---|---|---|---|
| **Identity** | **Karyology** | | DCL | MCB | | | |
| | | | | WCB | | | |
| | **Molecular Tests** | | DCL, CCL | MCB | | | |
| | | | | WCB | | | |
| **Cell growth profile** | **Viability** | | DCL,CCL | MCB | | | |
| | | | | WCB | | | |
| | **Growth Characteristics** | | DCL, CCL | MCB | | | |
| | | | | WCB | | | |
| | **Homogeneity** | | DCL, CCL | MCB | | | |
| | | | | WCB | | | |
| **Tumorigenesis** | **Tumourigenicity** | | DCL, CCL | MCB | | | |
| | | | | WCB | | | |
| | **Onchogenicity** | | DCL, CCL | MCB | | | |
| | | | | WCB | | | |
| **Stability** | **Genetic Stability** | | DCL, CCL | MCB | | | |
| | | | | WCB | | | |
| | **Stability during cryostorage** | | DCL, CCL | MCB | | | |
| | | | | WCB | | | |
| **Purity/ Sterility (Microbial agents)** | **Virus** | *Tests in animals and eggs* | DCL, CCL, PCC | MCB | | | |
| | | | | WCB | | | |
| | | *Tests in cell culture* | DCL, CCL, PCC | MCB | | | |
| | | | | WCB | | | |
| | | *Tests for particular viruses* | DCL, CCL, PCC | MCB | | | |
| | | | | WCB | | | |
| | *Bacterial and Fungal Sterility* | | DCL, CCL, PCC | MCB | | | |
| | | | | WCB | | | |
| | **Mollicutes** | *Mycoplasma* | DCL, CCL, PCC | MCB | | | |
| | | | | WCB | | | |
| | | *Mycobacteria* | DCL, CCL, PCC | MCB | | | |
| | | | | WCB | | | |

MCB: Master Cell Bank WCB: Working Cell Bank PCC: Primary Cell culture

CCL: Continuous Cell Line DCL: Diploid Cell Line

Comments:

Prepared By: Approved By:

This issue is very important when using a new cell and replacing it with the previous cell. If we have a correct strategy to identify, characterize, document, and control the cell, we can use that cell to produce and control a biological product, because using an inappropriate cell or not having the right program to have a cell in mass volume can cause irreparable damage to the production process. Therefore, manufacturers must have a specific strategy for cell preparation, cell identification and determination, cell documentation, and the use of controlled cells for different production processes. Therefore, in this review article, this strategy is drawn in a specific and codified form to be a road map for cell culture industries and laboratories in this direction. To be under the control of a cell, the set of general information as well as the specific information of the cell must be prepared. General information about the cell includes basic characteristics of the cell, cell type, cell storage information, coding, cell labeling, and cell passage information. Information about cell passage plays a key role in the suitability of that cell for use, especially in the production of biological products, and ensuring the correct passage and in the number of controlled passages. This information must be fully specified, collected, and edited (42). The specific information of the cell is the result of identifying and determining the characteristics of the cell in the laboratory. According to the WHO standard, a cell line to be used for the production of biological products must be examined and evaluated in various ways. After choosing a cell line, it should be studied and investigated in terms of growth characteristics, viability, identity, stability, homogeneity, cytogenetics, tumorgenicity, bacterial and fungal contamination, and other possible adventitious agents, especially viral infections (43). Describing the characteristics of the cell is vital not only to determine its capabilities but also to prove its authenticity, and based on the desired goal, the investigation and identification of the cell with various techniques such as karyotype, isozyme analysis, investigation of cell surface antigens and other methods are carried out. In the following, in order to use the desired cell in the biological products production industry, complete documentation must be prepared based on the studies conducted on the cell. The most important part of the cell's documentation is the cell's certificate, which must be prepared and controlled in two phases based on the general information of the target cell as well as the results of the tests performed on the cell. Based on the prepared certificate, it can be concluded whether the desired cell can be used, especially in the biological product production industry. In this review article, in three steps, the cell used for the production and quality control of biological products is identified, characterized, and documented. As a result, for the Identification process, all aspects related to early cell identification are discussed in this paper, including cell general information, cell specifications, cell storage conditions, and coding and labeling of the cells. All the said aspects were explained and discussed in this study. For the next step, the cell identification process was discussed. In this part, all the items that are needed to determine the characteristics of a cell for use in the production of biological products were explained in detail, including karyotyping, molecular tests, cell counting, viability test, cell growth characteristics, homogeneity, tumorgenicity test, oncogenicity test, study of genetic stability, study of stability during storage, evaluation of bacteria and fungi in cells, evaluation of adventitious viruses in cells, evaluation of mycoplasmas, and detection of mycobacteria. In the third step, an example of cell certificate includes all the identification and characterization has been shown. Furthermore, in the documentation section, other documents required for a cell in addition to the certificate of the cell were discussed. In this way, complete information for identifying, characterizing, documenting, and being under the control of a cell for using that cell in research and diagnostic studies in virology laboratories and especially for the production and quality control of biological products in the biological product industries are presented in this article. In this way, by using this strategy and possessing a suitable cell reserve of controlled cells, the many concerns existed regarding the use of these cells, especially in industries and among manufacturers, will be solved to a large extent. The key point in this article is that, a specific strategy has been drawn with an emphasis on identifying and determining cell characteristics so that the cells used in laboratories and industries can be properly examined and controlled. Considering that the main focus of this article is on the identification and evaluation of cells, some aspects affecting the cells, especially during the formation of the cell bank, are not included in this article, which need further studies.

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**Authors' Contribution**

Study concept and design: Soleimani S.
Acquisition of data: Soleimani S.
Analysis and interpretation of data: Soleimani S.
Drafting of the manuscript: Soleimani S.
Critical revision of the manuscript for important intellectual content: Soleimani S.

Statistical analysis: Soleimani S.
Administrative, technical, and material support: Soleimani S.

## Ethics
We hereby declare all ethical standards have been respected in preparation of the submitted article.

## Conflict of Interest
The authors declare that they have no conflict of interest.

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## Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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