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Practical Approaches to Improve the Sensitivity of the Volume-Based Cellular Enumeration of the BeWo Cell Line-Derived Spheroids as an *In Vitro* Embryo Model: MTT Versus Neutral Red Uptake Assays

Mahsa Kazemi[1,2], Vahid Jajarmi[3,4*], Hamid Nazarian[2], Marefat Ghaffari Novin[2], Saghar Salehpour[5], Hamid Choobineh[7], Zahra Shams Mofarahe[2], Mohammad Hasan Heidari[2*]

Abstract

Objectives: Choriocarcinoma spheroids, as acceptable three-dimensional (3D) models of the embryo, vary in the number of incorporated cells based on the procedures of production. Since the secretion of emitted signals from spheroids can modulate endometrial confrontation responses, improving a high throughput standard assay is precisely demanding for normalizing the characteristics of spheroids based on cellular enumeration.

Materials and Methods: Spheroids derived from the suspension culture of the BeWo cell line were dimensionally categorized into three groups with sizes of 50, 100, and 200 µm in diameters. The volume of each spheroid was determined according to $4/3\pi r^3$ formula and MTT and neutral red uptake (NRU) assays were applied as conventional and modified procedures. Furthermore, the categorized groups of spheroids were enzymatically dissociated and stained by the Trypan blue dye to precisely enumerate the number of incorporated cells based on volume alterations.

Results: The absorbance of spheroids in modified MTT and NRU procedures with respect to the conventional ones resulted in higher correlation coefficients with volume alterations. Similarly, modified MTT and NRU procedures with respect to the conventional ones resulted in higher correlation coefficients between the cells numeration and volume alterations. Finally, the sensitivity of modified MTT assay was higher than modified NRU assay regarding accurately estimating the number of incorporated cells per spheroid.

Conclusion: In general, the modification of MTT and NRU procedures can amplify the potential of both assays to enumerate the cell number of 3D aggregates. Exclusively, the modification of MTT assay brought it as an ideal high throughput assay with high sensitivity to screen the cell number of spheroids with a diameter of 200 µm and less.

Keywords: Cellular Spheroids, Choriocarcinoma, Neutral red, MTT formazan, Embryo implantation

Introduction

Human embryo implantation, as a charismatic *in vivo* condition, faces ethical limitations for precise experimental examinations (1). The quantitative systematic investigation for understanding complex cross-talks between the human embryo and endometrium through the coordinated process of implantation is impossible by the available spare *in vitro* fertilization embryos (1). To overcome this problem, various *in vitro* models were developed to virtually mimic an *in vivo* condition. For example, choriocarcinoma derived spheroid was established as an acceptable *in vitro* three-dimensional (3D) model system (2-7). The choriocarcinoma cell lines (BeWo, Jeg-3 and Jar cell lines), as the malignant counterparts of trophoblast, have characteristic similarities to the trophoblastic part of an embryo (1,8). Specifically, the BeWo cell line, as a heterogenic cell line with two populations of cells that differ in proliferation abilities (1), is sophisticated in specific trophoblastic characteristics that can motivate responses in the epithelial and stromal cells of the endometrium (2,4,5,9). Given that the maximal growth of a human embryo at the time of implantation is around 200 µm in diameter, choriocarcinoma spheroids with a size of 200 µm or smaller are assumed as ideal dimensions for mimicking the human embryo geometry (3,8). It is worth noting that the proliferative heterogenicity of

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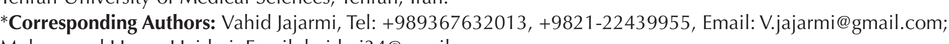
[1]Student Research Committee, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. [2]Department of Biology and Anatomical Sciences, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. [3]Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. [4]Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. [5]Infertility and Reproductive Health Research Center, In Vitro Fertilization Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. [6]Department of Obstetrics and Gynecology, Preventive Gynecology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. [7]School of Allied Medical Sciences, Tehran University of Medical Sciences, Tehran, Iran.

*Corresponding Authors: Vahid Jajarmi, Tel: +989367632013, +9821-22439955, Email: V.jajarmi@gmail.com;
Mohammad Hasan Heidari: Email: heidari34@gmail.com



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the BeWo cell line can cause variations in the number of incorporated cells in spheroids despite dimensional similarities (1). It is also shown that the overall growth of BeWo spheroids is due to both actual hyperplasia and some degree of hypertrophy (1). In addition, 3D culture strategies are commonly chosen based on cell line adaptability and expectance to resemble an *in vivo* condition (10,11). The cost-effective and high throughput suspension culture, as a routine strategy for producing a 3D spheroid in implantation experiments, is based on the self-aggregation ability of the cells which are seeded on a non-adherent surface (1,8). This production strategy can result in small cavities inside the BeWo spheroids which are devoid of cells as well. Caveated structures within spheroids can further challenge the accurate estimation of the cell number in each devoted volume morphologically (1). Furthermore, cellular density within the microenvironment of spheroids by influencing cell-cell communication can change the functional and behavioral attributes of 3D aggregates (12,13). Specifically, the modulated expression of the genes can lead to an altered emitted secretory profile of spheroids (11,14). Considering that multiple measurements of various derived factors are possible in the supernatant media of spheroids (8,15,16), the normalization of all quantifications based on the cellular enumeration of each spheroid seems necessary for interpreting a virtual *in vitro* response according to a real *in vivo* condition.

The histological estimation of the accurate cell number in the spheroid with a diameter of 200 µm and less is time-consuming and laborious (1). Viability assays are routinely applied to enumerate two-dimensional (2D) cellular density in toxicity assessments (17,18). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay (MTT) as a gold standard is based on the activity of the mitochondrial succinyl Coa enzyme (17). Moreover, the neutral red uptake (NRU) assay, which is based on the lysosomal accumulation of the dye, is another reliable assay for measuring viability and cellular density at 2D status (18). Although the MTT assay has recently been applied in cytotoxicity experiments on 3D cellular aggregates, the efficiency assessment of the established conventional procedure of MTT to precisely enumerate cellular density was not the scope of these experiments (14,19). Given that there is no established standard procedure of cellular enumeration based on viability assays on 3D cellular aggregates, the present study sought to apply the conventional procedures of MTT and NRU assays in parallel with modified procedures in order to approach a sensitive strategy to estimate the cellular enumeration of spheroids according to volume alterations.

Materials and Methods

This *in vitro* experimental study modeled preimplantation embryo by the suspension culturing of the BeWo choriocarcinoma cell line.

Monolayer Cell Line Culture

The BeWo cancer cell line (Choriocarcinoma, human, ECACC 86082803, Sigma-Aldrich) was cultured in Dulbecco's modified Eagle's medium (DMEM/Ham's F-12 medium). The medium was prepared from L-glutamine contained powder (Gibco Life Technologies Limited) and was supplemented with sodium bicarbonate, 10% (v/v) heat-inactivated fetal bovine serum (FBS), along with 100 U/mL penicillin and 100 µg/mL streptomycin sulfate. The cells were then cultured at 37˚C in a humidified 5% CO_2 incubator.

Three-Dimensional Cell Culture as Suspension Culture

The BeWo cell line was harvested by dispersion with 0.25% trypsin/ethylenediamine tetra-acetic acid (Gibco Life Technologies Limited) at 37˚C for 5 minutes. Then, the cell count of dissociated cells was determined to generate spheroids. For static culture, T75 culture flasks (SPL Life Sciences Co., Ltd.) were coated with 0.5% agarose that was dissolved in the DMEM media. Next, 2×10^6 BeWo cell was seeded in each coated flask that contained a 10 mL complete medium. After 60 hours of static culture, the flasks were placed on the gyratory shaker at 37˚C at 70 rpm for extra 12 hours. About 1 mL of Complete DMEM-F12 media was added to the flask every other day to supply nutritional requirements.

Imaging and Volume Analysis

For live imaging, morphologically spheroids with 50, 100, and 200 µm in diameters were categorized into three groups with 10 spheroids in each. The selected spheroids were separately placed in the wells of culture plates. Moreover, the phase-contrast imaging and measurements of spheroid diameters (µm) were carried out with a Nikon-Eclipse phase contrast microscope (TS100, Nikon, Japan) and the images were captured using the digital sight DS-K 10589 camera (Nikon, Japan). It should be noted that the spheroid volume calculation relied on the following equation.

$$V = 4/3\pi r^3$$

where r was the geometric mean radius of two orthogonal diameters.

Trypan Blue Cellular Enumeration

To calculate the average number of incorporated cells in each categorized group of spheroids, a mixture of cell suspension was prepared by trypsinizing the selected spheroids and then it was stained with trypan blue dye (Sigma). Next, the counted cells were averaged according to the volume of each categorized group of spheroids.

Viability Assays

Both MTT and NRU assays were carried out on both monolayer and three categorized groups of spheroids

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according to conventional and modified protocols. The modifications were settled according to a procedure which was represented in the commercial 3D Spheroid Colorimetric Proliferation/Viability Assay Kit produced by Trevigen Incorporation.

MTT Assay

Conventional Procedure

In brief, 20 µL of MTT solutions (5 mg/mL) was added to cells or spheroid-containing wells after 6-hours of incubation. According to the adjusted protocol by Ho et al, the plate was centrifuged to sediment the spheroids (19). After the aspiration of 50 µL of extra solution, the remaining solution was blotted by inverting the plate onto paper towels. Then, 100 µL of the acidic isopropanol (1.5% (*v/v*) solution of hydrochloric acid in isopropanol was added to each well to dissolve formazan crystals. Then, the absorbance at 570 nm was recorded using a multi-mode reader (BioTEK, synergy/HTX).

Modified Procedure

According to mentioned MTT procedure in the Cultrex® 3D Spheroid Colorimetric Proliferation/Viability Assay kit (Catalog #: 3511-096-K, Trevigen, Inc.), one-tenth volume (10 µL per 100 µL) of the MTT reagent was added to each well of the plate. The plate was transferred back to the 37°C incubator for 24 hours. There was no need to aspirate the extra solution before adding the detergent regent. Acidic isopropanol, as the detergent reagent, was warmed at 37°C for 5 minutes and then added to the plate (100 µL to each well). Next, the plate was transferred back to the 37°C incubator for another 24 hours and the absorbance at 570 nm was recorded using the multi-mode reader (BioTEK, synergy/HTX).

Neutral Red Uptake Assay

Neutral red, as a supravital dye, can incorporate and binds to viable cells. The cationic properties of the dye let its penetration through the cell membrane by nonionic passive diffusion. Additionally, the dye binds to anionic and/or phosphate groups of the lysosomal matrix and concentrates in the lysosomes (20,21).

Conventional Procedure

In general, the neutral red medium (40 µg/mL) was added to the cell or spheroid contained wells based on the established nature protocol (18). After 3 hours of incubation, destain solution (50% ethanol 96%, 49% deionized water, and 1% glacial acetic acid) was added and the absorbance was recorded at 540 nm using the multi-mode reader (BioTEK, synergy/HTX).

Modified Procedure

Similarly, the neutral red medium (40 µg /mL) was added to each cell or spheroid-containing wells. The plate was transferred back to the 37°C incubator for 24 hours. Further, the plate was centrifuged to sediment spheroids. After the aspiration of 50 µL of the extra solution, the remained solution was blotted by inverting the plate onto paper towels. The destain solution (50% ethanol 96%, 49% deionized water, 1% glacial acetic acid) was added and the plate was transferred back to the 37°C incubator for another 24 hours. Finally, the absorbance at 540 nm was recorded using the multi-mode reader (BioTEK, synergy/HTX).

Statistical Analysis

Three independent experiments with a minimum of ten internal replicates were performed and the data were assessed for normality using the Kolmogorov-Smirnov test. All results, presented as mean ± SD, were assessed with one-way ANOVA and Tukey post hoc tests. In addition, the linear correlation test was performed on the categorized groups of spheroids to further quantify correlation confidences between size variation and either absorbance or cellular enumeration. The results were noted as R^2 values and the analysis was carried out with SPSS software, version 16 (SPSS, SPSS Inc., USA).

Results

To optimize the sensitivity of MTT and NRU assays on volume-based cellular enumeration in the spheroids with a diameter of 200 µm and less, both tests were applied as conventional and modified procedures. Further, the accuracy of predicted cell density in both assays was verified by precise trypan blue-based cell count per dissociated spheroid.

Correlation Between the Quantified Absorbance of MTT Procedures and the Volume Alterations of Spheroids

Conventionally, formazan crystals were clearly shaped after 6 hours of incubating spheroids with the MTT solution. Descriptively, the punctuated pattern of provided crystals (Figure 1A) raised the doubt of insufficient penetration of the solution to the depth of spheroids. Contrary to this observation, the modified MTT procedure not only resulted in the complete penetration of the solution to the whole part of spheroids but also provided protruded highly assembled crystals that extended outward from the surface of the spheroids (Figure 1B). The standard curves of cellular enumeration based on two MTT procedures on the 2D status of the cells showed that the modified procedure resulted in higher absorbance per counterpart cell density (Figure 1C). In parallel, the data in Figure 1D demonstrated the absorbance of conventional and modified procedures of MTT on 3D spheroids. Although a similar ascending trend was observed in categorized spheroids within 50 and 100 µm in diameters, the absorbance of categorized spheroids within 200 µm in diameter represented a significant difference between conventional and modified procedures. The correlation coefficients of the modified procedure were higher

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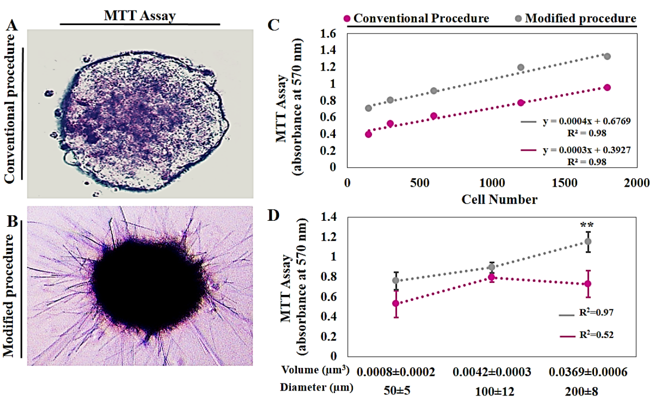

Figure 1. Conventional and Modified MTT Assays on BeWo-derived Spheroids.
Images **A** and **B** represent the descriptive feature of produced formazan crystals within spheroids as conventional and modified MTT procedures. In addition, image **C** displays the standard curve of determined cellular densities of the MTT assay as conventional and modified procedures. Finally, image **D** depicts the absorbance of categorized spheroids with the sizes of 50, 100 and 200 µm in diameters as conventional and modified MTT procedures. Data are shown as mean ± SEM (**P<0.01).

compared to the conventional procedure.

Correlation Between the Quantified Absorbance of NRU Procedures and the Volume Alterations of Spheroids

Descriptively, 6 hours of incubating spheroids with the dye resulted in accumulated neutral red punctuates (Figure 2A). The non-uniform distribution of neutral red punctuates in the spheroids can be speculated as either nonhomogeneous accumulation or the insufficient penetration of the dye to the depth of spheroids as well. As is shown in Figure 2B, the long-term incubation of spheroids with the neutral red solution provided a high density of dye accumulation in the cells. Further, the standard curves of cellular enumeration at the 2D status of the cells showed that the modified procedure of NRU with respect to conventional one led to higher absorbance per counterpart cellular density (Figure 2C). Figure 2D represents the absorbance of conventional and modified procedures of NRU on 3D spheroids. Although the absorption was similar in categorized spheroids within 100 µm in diameter, the trends of the spheroid absorbance based on conventional and modified NRU procedures demonstrated a significant difference between categorized spheroids within 50 and 200 µm in diameters. Eventually, the correlation coefficient of the modified NRU procedure was higher than that of the conventional procedure.

Correlation Between Cell Density Estimation of Either NRU or MTT Assays and Trypan Blue Based Cellular Enumeration of Spheroids

Precise cellular enumeration in each categorized diameter of spheroids was applied on dissociated spheroids based on trypan blue staining. The cell density of each categorized group of spheroids was also predicted based on the

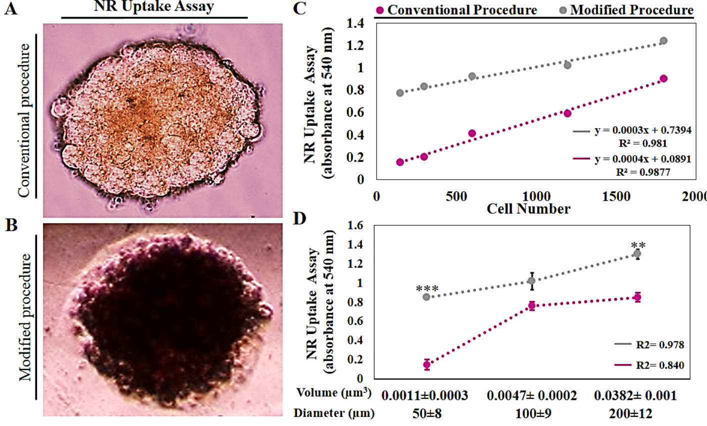

Figure 2. Conventional and Modified NRU Assays on BeWo-derived Spheroids.
Images **A** and **B** represent descriptive features of the accumulated neutral red dye within spheroids as conventional and modified NRU procedures and image **C** illustrates the standard curve of determined cellular densities of the NRU assay as conventional and modified procedures. Furthermore, image **D** displays the absorbance of spheroids with the sizes of 50, 100, and 200 µm in diameters as conventional and modified NRU procedures. Data are shown as mean ± SEM (**P<0.01 & ***P<0.001). *Note*. NRU: Neutral red uptake.

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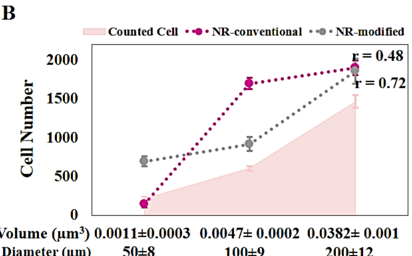

Figure 3. Calculated Cellular Density of Spheroids Based on MTT and NRU Assays in Accordance With Trypan Blue Cellular Enumeration.
In image **A**, the dashed lines represent the calculated cellular enumeration based on volume alterations according to the conventional and modified procedures of the MTT assay. Moreover, the pink area displays the average counted cells of 10 dissociated spheroids based on Trypan blue staining and the correlation is indicated as r coefficients. Additionally, dashed lines in image B depict the calculated cellular enumeration based on volume alterations according to the conventional and modified procedures of the NRU assay. In addition, the pink area represents the average counted cells of 10 dissociated spheroids based on Trypan blue staining. Data were presented as mean ± SEM and the correlation is demonstrated as r coefficients. *Note*. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NRU: Neutral red uptake.

achieved line equation (Figures 1C and 2C). According to Figures 3A and 3B, modified MTT and NRU procedures with respect to conventional ones resulted in higher correlation coefficients between cell density prediction and trypan blue-based cellular enumeration. However, the modified MTT assay showed a 0.99 correlation coefficient and the modified NRU assay demonstrated only 0.72 correlation coefficient with cellar density estimation of each spheroid.

Discussion

Choriocarcinoma cell line-derived spheroids have been accepted as an applicable model system for studying the implantation process (2-7). Further, the aggregation of the spheroid in the suspension culture is considered as a complex molecular process which is primarily resulted from cell-cell attachment through the expression of the cadherin (22,23) and is further stabilized with the ligation of integrins and cytoskeleton proteins (22,24-26). Furthermore, the cyto-architecture of provided spheroids based on morphophysical characteristics such as structural rigidity and compaction (10,11) can result in the cytoskeleton dependent activation of intracellular signaling cascades (22,24,27,28). Hence, the density of the incorporated cells in the microarchitecture of the spheroids can affect the expression and translation of various genes and proteins by re-arranging the cellular cytoskeleton (9,11,14,29-32). Moreover, the profile of spheroid derived factors (e.g., hormones, growth, and immunological factors) results in specific responses in the endometrium (33-35). Consequently, the characterization of derived factors based on the cellar enumeration may help the accurate interpretation of cellular and molecular mechanisms of an *in vitro* model of virtual implantation.

Viability assays such as NRU and MTT have been vastly employed for screening the cells at the 2D status (12). Alive cells are able to uptake and accumulate the neutral red within cellular lysosomes (12) while the water-soluble tetrazolium salt of the MTT assay is converted to insoluble purple formazan crystals by the mitochondrial succinate dehydrogenase activity (13,19). Although there is not any consensus about the standard procedures of MTT and NRU assays on spheroids, their simplicity and rapidity, which can be carried out in multiwall plates (19), have made them as appropriate candidates for screening the cellular enumeration of spheroids. Conventionally, both MTT and NRU assays contain three main steps including incubation with staining solutions that is reported from 3 to 6 hours, as well as the aspiration and blotting of the extra solution and the solubilization of the aggregated dyes to record the absorbance (12,18,19,36). Although a recent experiment with some modifications in the aspiration and blotting of the plate made the procedure of MTT assay applicable for the cytotoxicity assessment of spheroids (19), it needs to emphasize that no data are available about precise cellular enumeration. Based on our presented data, it can be clearly concluded that the conventional procedures of both MTT and NRU assays were not efficient for estimating cellular density based on volume alterations. A reasonable justification for this observation can be attributed to the 3D architecture that can result in unique cellular, along with morphological and physicochemical gradients across spheroids (37-40). This phenomenon can adversely challenge the reproducibility of cellular enumeration based on conventional procedures of viability assays by reducing the penetration ability of the dye into the depth of spheroids. To reiterate its importance, it is well defined that the uptake and lysosomal aggregation of the neutral red is closely related to the pH gradient and a net charge of the cellular membrane (18). Hence, the culture condition of spheroids can affect the results of the assays by modulating important modalities (37-40). Moreover, the compaction and rigidity of the spheroid structure may interfere with the complete solubilization step of the procedure and result in biased absorption.

The presented modified procedures of both MTT and NRU assays not only increased the chance of dye absorption by prolonging the incubation time but also provided a vast time for the solubilizing detergent to release all accumulated dyes from 3D cellular aggregates.

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Specifically, the lack of the need for extra MTT solution aspiration and blotting the plate made the technique user-friendly and much more applicable to spheroids with a diameter of 200 μm and less. Given that the surface of the cells can alter the uptake of the dye (18), the modification of both the enzymatic-based MTT assay and colorimetric-based NRU assay (12) increased the chance of penetration and extra accumulation of dyes in the whole depth of spheroids by providing cell surface availability. Additionally, not only the modified procedures of MTT and NRU assays provided high correlations between the absorbance and volume alteration of spheroids but also the accuracy of the modified procedure for estimating cellular enumeration based on the volume alteration of spheroids were higher compared to conventional procedures. Notably, a 0.99 correlation coefficient of the modified MTT procedure acknowledged its superiority rather than the modified NRU procedure. The difference between the sensitivity of viability assays on cellular enumeration can be attributed to the lysosome-based colorimetric quiddity of the NRU assay. It was well notified that lysosomal function may result in a discrepancy in estimating the cellular density by interfering in the accurate reflection of cell proliferation (18). Considering that the present data were obtained based on the aggregating ability of one cell line based on a single production strategy, this experiment needs to be re-implemented on various cell lines with different production strategies for further affirmation. The specificity of difference viability assays on enumerating the cell density at the 2D status was reported previously (37). Hence, further viability assays such as protein-based quantification seem worthful for further assessments.

Conclusions

In general, BeWo spheroids are accepted as the *in vitro* model of embryo implantation. Given that the procedure of spheroid production can alter the number of incorporated cells within the spheroids albeit of volume similarities, developing a high throughput screening procedure that enumerates cellular incorporation within spheroids paves the way for mimicking an *in vivo* condition based on fine-tuned *in vitro* emitted signals. In addition, the conventional MTT assay with some modifications brought the hope of its application on the cytotoxicity assessment of multicellular spheroids. In this study, the modification of MTT and NRU viability assays provided desirable sensitivity for precisely predicting the cell density within each spheroid. Notably, the modified MTT assay showed its accurate superiority rather than the NRU one for accurately predicting the number of the incorporated cell per spheroid with a diameter of 200 μm and less.

Conflict of Interests

Authors have no conflict of interests.

Ethical Issues

The experimental procedures were accomplished upon obtaining permission from the Ethical Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (the ethical code: IR.SBMU.MSP.REC.1396.235).

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