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Research Article
Cytotoxic Evaluation Using Murine Fibroblasts (L-929) Three Dimensional Cell Culture Technique

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Abstract

Background and Objective: Cell based assays are simple tools with utilizing the conventional monolayer mode, but the absence of a real cell environment required an alternative technique to bridge the gap. This study was aimed to evaluate the possible application of three-dimensional cultures for cytotoxic testing. **Materials and Methods:** Three-dimensional (3D) cultures of murine fibroblasts or L-929 cells were grown by seeding a fixed density (1000 cells/well) in a 96 well plate coated with 1% w/v agarose. The spheroids were observed through different magnifications to observe its cellular structure. These cultures were treated against different types of toxicants and the reactivity was assessed qualitatively and quantitatively via XTT assay. Numerical data was calculated Kruskal Wallis-ANOVA followed by Dunnett *post hoc* for statistical differences. **Results:** Imaging of these fibroblastic spheroids at 1000 cells/well density at different magnifications exhibited; compact spheroids, change in cell morphology and increased cell-to-cell interaction, indicating a similarity between the spheroid and an *in vivo* environment. Upon exposure with the zinc dibutyldithiocarbamate (ZDBC) powder, Sodium Lauryl Sulfate (SLS) and extract of ZDBC film and latex glove, the percentage cell viability was calculated to be directly proportional with the concentration of these compounds. **Conclusion:** The results confirmed the required sensitivity and specificity that can be used for biocompatibility testing.

Key words: Murine fibroblast, cytotoxicity, sodium lauryl sulfate, zinc dibutyl dithiocarbamate, cell viability, latex glove, biocompatibility testing

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Biomaterials or any packaging material fabricated come into direct or indirect contact with living tissues[1]. Therefore, before their market release, all such manufactured materials must go through a comprehensive and fastidious testing to assess their conceivable toxic action by utilizing different *in vitro* and *in vivo* experiments[2].

In accordance to guidelines of United States Pharmacopoeia (USP 87)[3] and International Organization of Standardization (ISO 10993 Part 5)[4], these cytotoxic assays are classified as; direct contact between target cells and materials and indirect contact using a barrier between cellular monolayer and the material[1,3,4].

Currently, there was an exponential increment in the assembling of biomaterials, which may be dangerous so to maintain a strategic distance from such dangers, biocompatibility testing was obligatory for these materials[2]. In this context, assessment regarding the biocompatibility of materials through *in vitro* testing produces a biological response using a specific cell line in a specific environment[3,4]. In any case, the outcomes acquired from *in vitro* testing of materials are questionable and uncertain because of false positives or false negatives results.

For many decades, two-dimensional cellular layer or monolayer grown in a flat bottom cell culture vessels have been utilized for various *in vitro* assays[5]. However, because of their fixed and rigid structure, they cannot be compared to the physiological structures present in the *in vivo* assays[6]. Growing or culturing cells in a different type of cultures can mimic the micro-environment of the animal models and also prove to benefit for cytotoxic testing and drug screening[7].

The concept of 3D culture is based on the creation of spheroid structures in which cells form multiple layers and mimicking the physical and biochemical features of the physiological environment of a living organism[8]. Actually, the organisms are three-dimensional arrangement of cells with intricate cell-cell and cell-matrix interactions with complex transport dynamics for nutrients and cell inflow and outflow[9]. Cultured cells grown in a three-dimensional media provide a proper cell to cell and cell to environment interactions. Cells also can receive stimuli from the local environment[10].

The matrix based method was an effective way to grown cells in a three dimensional manner. The matrix used was 1% agarose, which employs the use of a 96 well plate pre-coated with it. The principle behind this method is to disable the cells from adhering to the flat surface of well and enable them to adhere to one another to form an aggregate then convert into a compact structure[11,12]. The application of three dimensional cultures in biocompatibility testing can provide a bridge between the *in vivo* and *in vitro*[13].

In lieu to cytoxicity and biocompatibility studies, the cell to material response was evaluated against toxicants such as; latex gloves, ZDBC (zinc dibutyl dithiocarbamate; film form) and Sodium Lauryl Sulfate (SLS), against the two-dimensional cellular layer. These compounds have proven to be severely cytotoxic in their undiluted form or at their highest concentrations[14]. So, therefore this study was aimed to investigate the structural aspects of a spheroid at higher magnifications and assessed the reactivity of various cytotoxic compounds such as; ZDBC (both film and powder), SLS and latex gloves at different concentrations against three dimensionally grown L-929 cells.

MATERIALS AND METHODS

Materials: This study was conducted at Vimta Lab Limited during February, 2017-February, 2018, 0.25% polyurethane ZDBC film was procured from Hatano Research Institute and ZDBC powder was procured from TCI chemicals, Sodium lauryl sulfate was procured from Merck and latex gloves from local vendors. All other chemicals were of commercial grade.

Cell culture: Murine fibroblasts cell lines, L-929 (American Type Culture Collection, ATCC) were suggested for use as per USP 87[4] and ISO 10993-5[3]. They were cultured, in complete Dulbecco's Modified Eagle's medium (DMEM). Prior to the initiation of the assay, the cells were grown to ~80% confluence and were performed at 37±1°C in a humidified atmosphere containing 5% Carbon Dioxide (CO_2) incubator.

Parameters calculated: Both the quantitative and qualitative parameters were performed in this study. The viability (%) was considered as the parameter for quantitative evaluation as per the ISO 10993 guideline using XTT assay, spheroid aggregate evaluation to assess the optimum seeding density for culturing spheroids and SEM imaging to better visualize the cell-cell interaction based on the Restle *et al.*[1], Costa *et al.*[15] and Ehrhart *et al.*[16].

Aggregate evaluation: For a homogenous 3D model cell culture model, L-929 cells were grown in agarose coated 96 well plate at a seeding densities of 10000, 5000, 2500 and

1000 cells per well with media change on alternate days. The structures were evaluated daily to determine which cell density would be adequate for this study[1].

Spheroid formation: The L-929 spheroids were cultured using the Liquid Overlay Technique (LOT). About 50 µL of 1% w/v volumes of agarose was prepared, coated and dried[15] for 20 min. This agarose formed a concave shape, thus preventing adhesion of cells to the well's surface. Cell suspension of volume of 100 µL was added to the agarose coated well for aggregate formation.

Scanning Electron Microscope (SEM) analysis: For SEM imaging, spheroids were fixed in Karnovsky fixative for 1 h followed by consequent PBS washings and dehydration using ethanol of different concentrations (30, 40, 50, 60, 70, 80, 90 and 100%) for 20 min. The samples were then dried with carbon dioxide and mounted on a stainless steel stub. The sample was viewed at 15 eV accelerating voltage using Hitachi SEM Table top[17].

Preparation of ZDBC powder: The stock solution of 250 mg mL^{-1} was prepared by dissolving the weighed amount of ZDBC powder in the required volume of DMSO. Final working concentrations of 0.25, 0.125, 0.0625, 0.03125, 0.01563, 0.007 and 0.003 mg mL^{-1} were prepared by diluting the secondary stocks with DMEM supplemented with 10% FBS. Each concentration along with vehicle control and blank were performed in triplicates. The DMSO concentration was maintained at 0.1% in all prepared concentrations.

Preparation of 0.25% polyurethane film and latex gloves extract: About 0.25% ZDBC polyurethane film and the latex gloves were extracted at a ratio of 6 cm^2 mL^{-1} in DMEM supplemented with 5% FBS for 24 h at 37±1°C, under dynamic conditions (120 rpm)[18]. This ratio was based on the thickness of the film's thickness, since its thickness is lesser than 0.5 cm^2. The 100% extract was diluted with media (5% FBS in DMEM) to obtain concentrations[19] of 50, 25 and 12.5% (latex glove extract) v/v.

Preparation of SLS concentrations: The initial stock of 10 mg mL^{-1} was prepared by dissolving SLS powder in 10% FBS in DMEM. The working stocks of 5, 2.5, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 mg mL^{-1} were prepared with 10% FBS in DMEM. Each concentration along with blank was performed in triplicates.

Cytotoxicity assay: In accordance with ISO 10993-5, XTT was used for quantitative analysis. The XTT/PMS was prepared (1 mg mL^{-1}); Phenazine methosulfate (PMS) was weighed and dissolved in PBS to obtain a concentration of 5 mM. The PMS solution was added to the XTT solution at a concentration[3] of 25 µM.

To 3D cultures, 50 µL of XTT/PMS solution was added to the 100 µL aliquot and incubated for 3 h at 37±1°C with 5% CO_2. After incubation, the plates were analyzed spectrophotometrically at 450 nm (Perkin Elmer Multiplate Reader) and viability (%) was calculated by comparing with blank:

$$\text{Viability}(\%) = \frac{\text{Optical density of test}}{\text{Optical density of blank}} \times 100$$

Statistical analysis: Experiment was carried out in triplicates and results were expressed in mean±standard deviation (SD). The percentage viability was calculated using Microsoft excel (2007) with Mean±SD. For statistical analysis, the percentage viability from the cytotoxicity assay of different compounds were tested for normality and a Kruskal-Wallis non-parametric one way analysis of variance was performed with a Dunnett post test comparing the concentration of the compounds, with an alpha error of 5%. The analysis was performed with the help of the software GraphPad Prism 4.0 (GraphPad, USA).

RESULTS

Aggregate evaluation: The L-929 cells were seeded at different densities to evaluate the quality of the aggregates. The higher densities (Fig. 1a: Pink arrow and 1b: Orange arrows) had formed compact structure, but of poor quality of aggregates. After 3 days, the aggregates grew in size, but failed to assemble into the required spheroidal form. The wells seeded with densities 2500 and 1000 cells (Fig. 1c, d), after 4 days had resulted into an ideal compact structure, however; 2500 cells/well seeding density failed to reproduce the same in all of its replicates. The well seeded with 1000 cells reproduced uniform structures throughout the wells with the three distinctive layers.

L-929 cells in 3D models

Morphological evaluation: Morphology of the cell changes depending on the construct utilized to culture them (Fig. 2). Figure 2a exhibited the conventional monolayer seeded in a

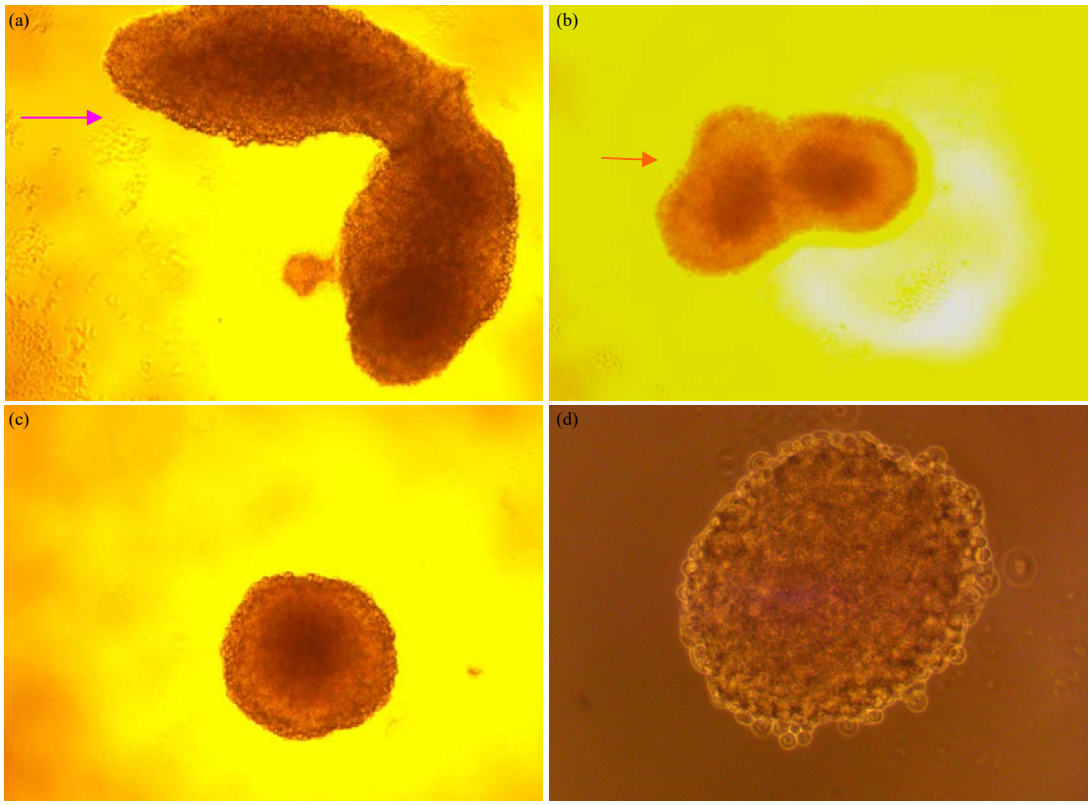

Fig. 1(a-d): L-929 cells were seeded at different densities in an agar coated 96 well plate to establish an optimum seeding density, after 4 days the spheroids were assessed for reproducibility and the essential layers, (a) Seeding density of 10,000 cells/well, the structure was irregular and distorted (pink arrow), (b) Cells were seeded at a density of 5,000 cells/well and the structure was distorted (red arrow), (c) Cells were seeded at a density of 2,500 cells/well and the structure resulted with a complete spheroid with the distinguishing layers and (d) Cells were seeded at a density of 1,000 cells/well and the structure was a complete spheroid

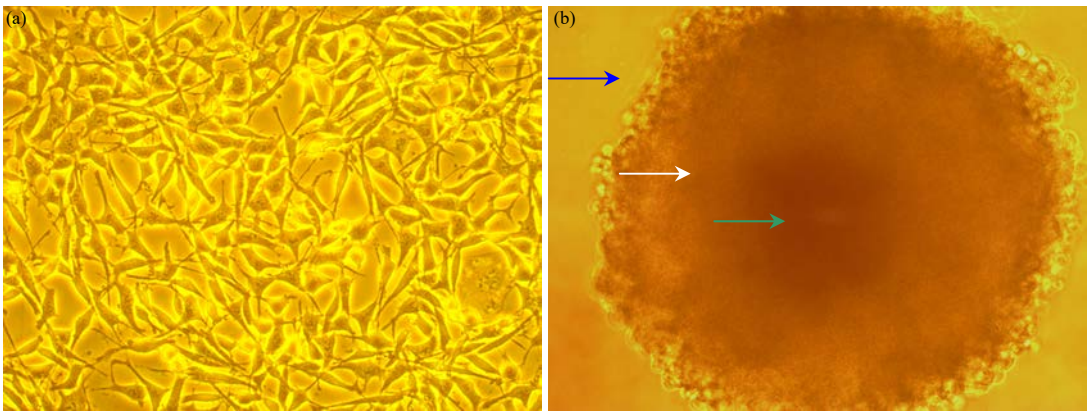

Fig. 2(a-b): L-929 spheroidal cultures were seeded at the same density, i.e., 1000 cells/well and grown in different constructs. They were observed under light microscopy at 10x objective, (a) The cells were seeded in a 96 well plate at density of 1000 cells/well representing a two-dimensional (2D) construct and (b) L-929 cells were seeded in a 1% agarose coated 96 well plate representing three-dimensional (3D) construct, where the dark blue arrow is representing the proliferating layer, white arrow is representing the quiescent layer and the green arrow represents the necrotic core

96 well flat bottom plate, where the flat morphology can be clearly observed, whereas (Fig. 2b) L-929 were seeded in a agarose coated well, which have exhibited a well connected network with three distinctive layers, i.e., proliferating layer (blue arrow), the quiescent layer (white arrow) and the necrotic core (green arrow). This network was verified by observing the spheroids under a scanning electron microscope (SEM). The L-929 spheroids have exhibited a uniform and compact surface when observed through the SEM as shown in Fig. 3a (500x) and 3b (400x). Upon enclosing in on the structure at a higher magnifications (Fig. 3c: Brown arrow) exhibited decreased space between cells the cell's morphology had been elongated, thereby improving cell to cell interaction (Fig. 3d, e: Yellow arrows).

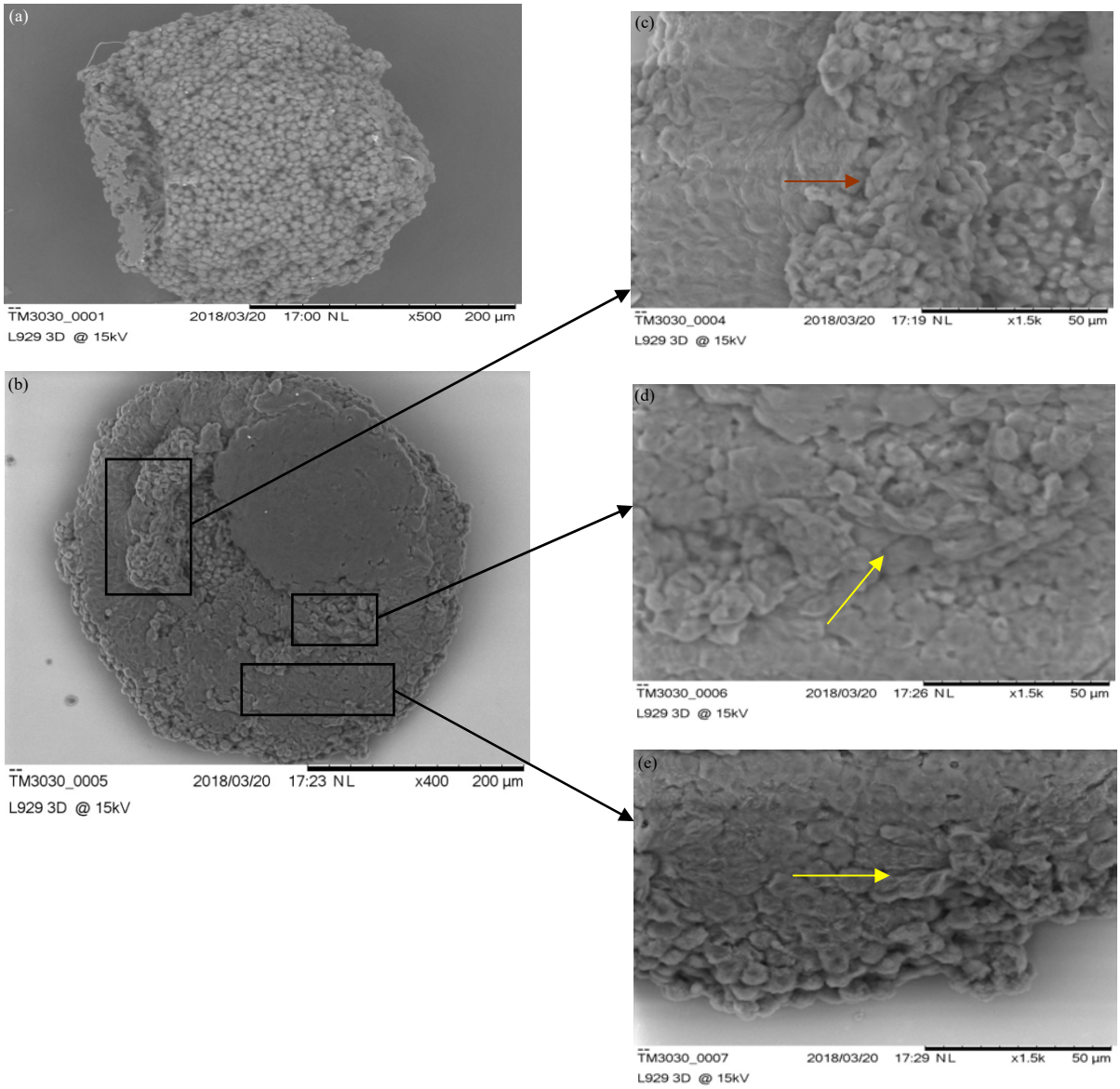

Fig. 3(a-e): Images of L-929 spheroids were taken using scanning electron microscope (SEM) at 15 eV at different magnifications, (a) SEM imaging of a whole spheroid, taken from a lateral view at 500x magnification, (b) SEM imaging of the L-929 whole spheroid, taken from a posterior view at 400x magnification, (c) SEM imaging of the zoomed section of the whole spheroid indicating the decreased spaces between the cells (brown arrow), (d) SEM imaging of the zoomed section of the whole spheroid indicating the elongated cell morphology (yellow arrow) and (e) SEM imaging of the zoomed section of the whole spheroid indicating the change in cell morphology (yellow arrow)

3D L-929 cellular

Cyto-compatibility evaluation: The interaction of biomaterials with the three dimensional (3D) system was analyzed using XTT assay.

ZDBC powder: Exposure to ZDBC powder at the concentrations; 0.25 mg mL^{-1} (Fig. 4a) have exhibited significant toxicity. Aside from that other concentrations such as; 0.125, 0.0625 and 0.031 mg mL^{-1} caused a severe cytotoxic effect as depicted in Fig. 4b-d. It was visible that the above concentrations had reduced the spheroid to cell debris, whereas the concentrations; 0.015 mg mL^{-1} had shown only cell detachment (Fig. 4e). The remaining concentrations; 0.007 and 0.003 mg mL^{-1} had shown no

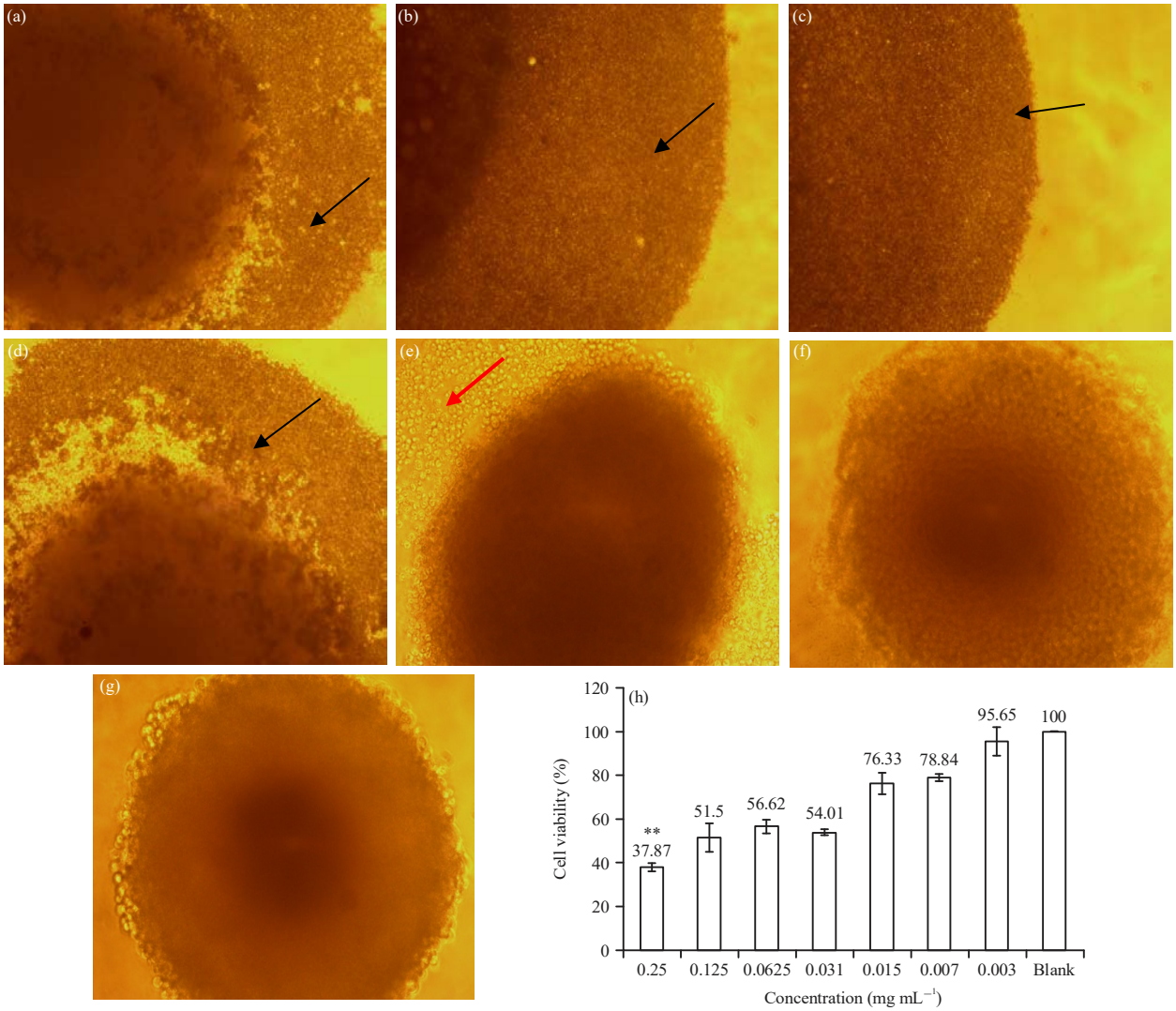

Fig. 4(a-h): ZDBC powder of different concentrations was treated against L-929 spheroidal cultures for 24 h, (a) L-929 spheroids were treated with 0.25 mg mL^{-1} ZDBC powder. This high concentration has exhibited cytotoxicity, (b) L-929 spheroids were treated with 0.125 mg mL^{-1} ZDBC powder has reduced the cells to debris (black arrows), (c) L-929 spheroids were treated with 0.0625 mg mL^{-1} ZDBC powder has reduced the spheroidal cells to debris (black arrows), (d) L-929 spheroids were treated with 0.031 mg mL^{-1} ZDBC powder and have reduced the spheroidal cells to debris (black arrows), (e) L-929 spheroids were treated with 0.015 mg mL^{-1} ZDBC powder and had detached the cells the cell from its compact structure (red arrow), (f) L-929 spheroids were treated with 0.007 mg mL^{-1} ZDBC powder and spheroid remained intact, (g) L-929 spheroids were treated with 0.003 mg mL^{-1} ZDBC powder and caused no cytotoxicity towards the cells and (h) The graph shows cell viability data of L-929 cells analyzed using XTT assay

Data are presented as the Mean±SD, **Significant toxicity with a cell population with that of Blank (n = 3)

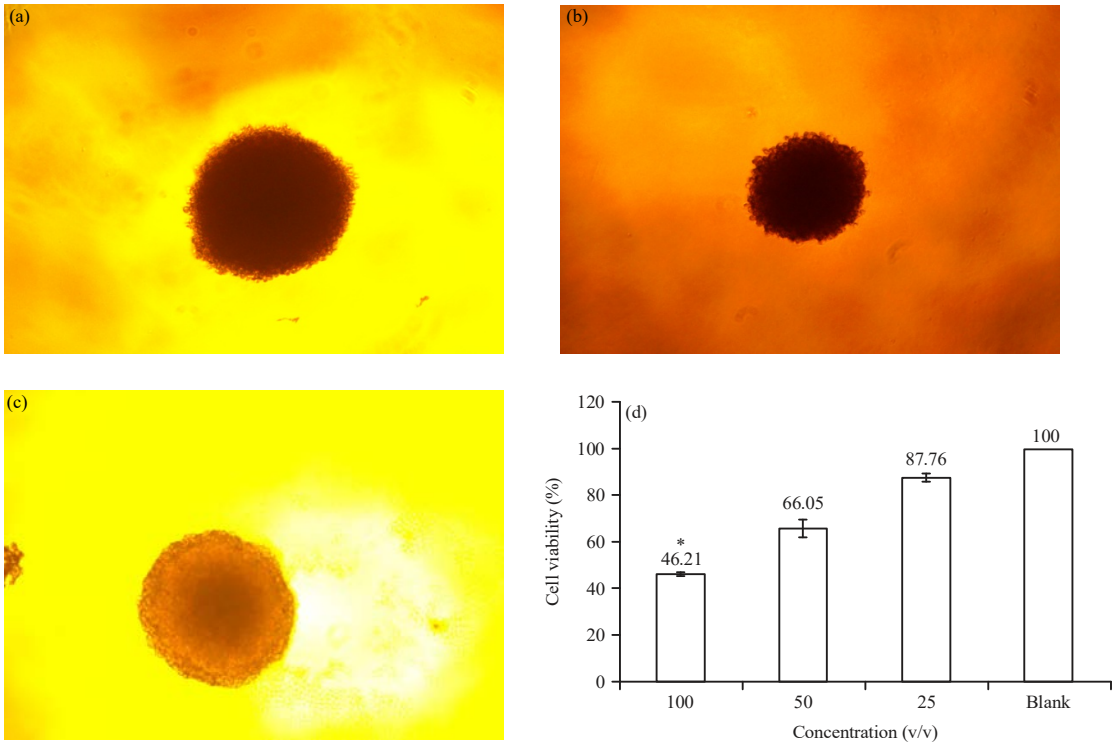

Fig. 5(a-d): Extract of 0.25% polyurethane ZDBC film concentrations was exposed to the L-929 3D cultures for 24 h, (a) 100% extract of the ZDBC film was treated against L-929 spheroid and exhibited cytotoxicity, (b) About 50% extract of the ZDBC film was treated against L-929 spheroid and exhibited cytotoxicity, (c) About 25% extract of the ZDBC film was treated against L-929 spheroid and exhibited no significant cytotoxicity and (d) The graph shows cell viability data of L-929 cells analyzed using XTT assay

Data are presented as the Mean±SD, **Significant toxicity with a cell population with that of Blank (n = 3)

effect on the spheroid (Fig. 4f, g). The qualitative imaging of the exposure ZDBC powder correlates with the percentage of cell viability performed through a quantitative analysis using XTT assay (Fig. 4h).

ZDBC film: Exposure to ZDBC film at various diluted concentrations; 100% extract, exhibited a significant cytotoxic effect (Fig. 5a). The diluted extract, i.e., 50% v/v was also exhibited cytotoxicity (Fig. 5b) and the last concentration 25% v/v has exhibited no cytotoxicity due to its intact structure (Fig. 5c). The percentage of cytotoxicity was quantified using XTT assay (Fig. 5d).

Sodium lauryl sulfate: The SLS has exhibited dose dependant cytotoxicity, where the concentrations 5, 2.5, 1, 0.5, 0.25, 0.125 and 0.06 mg mL^{-1} were exhibited severe cytotoxic reaction as their cell viability was lesser than 70%, out of which 5 and 2.5 mg mL^{-1} have shown significant toxicity. The last two concentrations (0.03 and 0.01 mg mL^{-1}) were exhibited no cytotoxic reaction (Fig. 6).

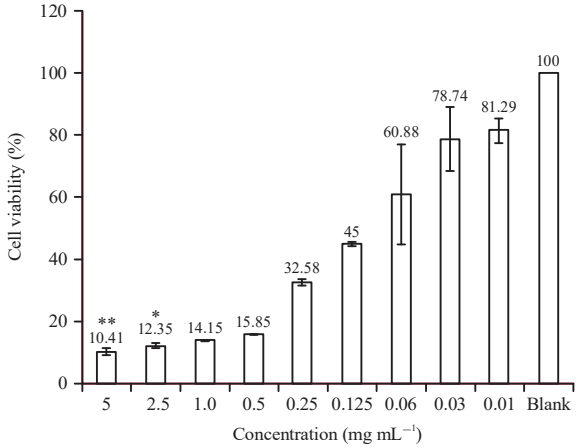

Fig. 6: Percentage viability of L-929 spheroids against various concentration of sodium lauryl sulfate

Graph shows the cell viability (values listed on top of the bars) and was analyzed using XTT dye, data are presented as the Mean±SD, significant toxicity (* and **) with a cell population with that of Blank (n = 3)

Latex glove extract: The 100% pure extract of the latex glove exhibited a significant toxicity and despite diluting

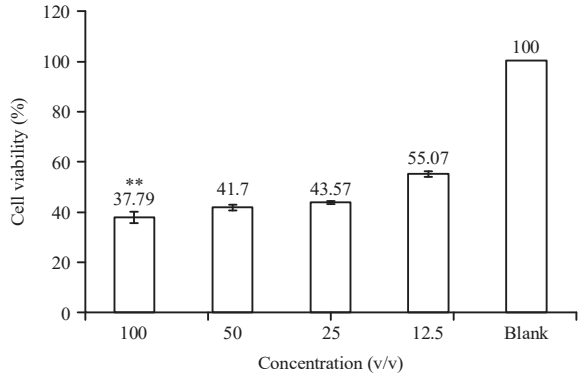

Fig. 7: Percentage viability of L-929 spheroids exposed with various concentrations of latex glove extract for 24 h
Graph shows the cell viability (values listed on top of the bars and was analyzed using XTT assay, data are presented as the Mean±SD, **Significant toxicity with a cell population with that of Blank (n = 3)

the extract 4 folds, the least concentration, i.e., 12.5% v/v had shown a cytotoxic reaction (Fig. 7).

DISCUSSION

In this study, fibroblast cells were grown in a three dimensional construct using the Liquid Overlay Technique (LOT) demonstrated an optimum seeding density at 1000 cells per well which were subjected to SEM imagining, showing a homogeneous compact structure with an increased cell to cell interaction. Up till now, there are very few studies reliably describing the use of the 3D model of the murine fibroblasts which were used vastly in the biocompatibility studies as a promising tool. As per the results of the XTT assay and the conclusion drawn regarding that the three dimensional cultures, they can be a promising tool for biocompatibility studies in agreement with the results and conclusions similar to those reported[1].

The results of the present study were supported by the declaration that the three-dimensional cultures are a proper model for cytotoxicity testing and to understand the cell to material interaction due to its similarity to the *in vivo* physiology[20,21].

The results of the current study showed that the ZDBC in both the powder form and the film forms exhibited higher cytotoxicity which were analogous for its positive control properties and this outcome was allied to the recent study of cytotoxic assessment of ZDEC (zinc diethyl thio carbamate) which was specified an alternative positive control in the regulatory guidelines[3,4] for all the biocompatibility studies against murine and human fibroblast cells[22].

The information regarding cytotoxicity of latex gloves extract using serum supplemented media contradicted when compared to the reported data of 2D culture which were showing a percentage of viability of less than 20% for the undiluted glove extract[19]. Therefore, the rising of such models which can differentiate the cytotoxic effect of the biomedical devises may lead to revolution in producing and overcoming with results which are more reliable in the field of the biomedical industry.

In a similar pattern, the results of the present study with the exposure of the sodium lauryl sulfate to L-929 spheroid cultures at various concentrations were found to be in contradiction with a previous study in which SLS resulted to be non-cytotoxic or higher cell viability at the least concentration against these spheroidal cultures in comparison to the conventional monolayer[23].

In vitro cytotoxic assays by means of the 2D cultures are used to envisage human toxicity; however, the drawbacks involving 2D cultures have increased drastically over the decades. The conventional model can in the future prove to be inadequate for cytotoxic testing and for drug screening. Aside from biocompatibility, various applications such as; drug toxicity screening[24], nano-carrier toxicity[25,26] etc. have also been reported using 3D cultures.

However, the method adopted in quantifying the cell viability in this study was through XTT assay. The results were prominent and consistent throughout the study with significant results, required data output and this model has provided evidence as a cost effective and simple technique for the biomedical research. By using optimized conditions from this study, further more studies can be required for employing the other quantification practice on 3D spheroids[22].

The relevance of three-dimensional cultures has grown tremendously in the last decade. Growing primary cell lines in a three dimensional construct can act as a more complex system for preliminary screening for pre-clinical studies in upcoming days[27]. Three-dimensional cultures can be applied in the cytotoxic evaluation and provided more information regarding to cell to material interaction and also reduce false results.

CONCLUSION

The SEM imaging concluded that cell to cell interaction in spheroids exhibited similarities in the cellular structure of that the *in vivo* physiology. Fibroblast spheroidal cultures can be used as a promising tool for biocompatibility studies, material screening and bridge the gap of the *in vivo* and *in vitro* 2D model.

SIGNIFICANCE STATEMENT

This study discovered an alternative method or a possibility of using spheroidal cultures and its advantages towards biocompatibility studies. By growing cells in a three dimensional construct, it would reduced any and all false results, decrease the need for animal testing and provide more information on cell to cell and cell to material interaction. Therefore, spheroidal culture can act as an alternative method for cytotoxic assays and drug screening assays using conventional cellular monolayer and perhaps bridge the gap between the *in vitro* and *in vivo* models.

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