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A MODIFICATION METHOD FOR EVALUATION THE IC50 FOR CHEMOTHERAPEUTIC EFFICACY ON 3D TUMOR SPHEROID

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ABSTRACT

The in vitro evaluation of chemotherapeutic agents using cellular monolayers (2D) cultured in a flat-bottomed plastic culture dish doesn't give the predictive responses similar to that found in vivo investigations, because of the lacking of cell–cell and cell microenvironment interactions in cellular drug responses. Hence, there are strong needs for the development of the in vitro 3D tumor models that closely mimic the in vivo microenvironment interactions with the surrounding cells, growth factors, and other biomolecules. To date, the sulforhodamine B assay still the most widely technique used for two dimensional (2D) cytotoxicity screening of the chemotherapeutic agents. Herein, this study has been optimized for establishing modification method based on growing the human tumor cell lines as three-dimensional culture (3D) tumor spheroids using hanging drop technique in flat-bottomed 96-well plates to make tumor spheroid into every well of plate. Here we study the differences biological responses of human colon cancer HCT116 cell line cultured as three dimensional (3D) spheroids compared to monolayers to doxorubicin which is used in treatment of a wide variety of cancers to compare the IC50% values between two types of culture.

KEY WORDS

Tumor spheroids, Chemotherapeutic agents, Modification method for evaluation of IC50.

INTRODUCTION

Two-dimensional (2D) *in vitro* cell culture systems have limitations; cells are exposed to an unnatural environment for cell growth, as 2D culture systems lack *in vivo* components including the extracellular matrix (ECM), signaling molecules, and stromal cells [1, 2].

Many types of mammalian cells can form three-dimensional (3D) multicellular spheroids. Compared to the traditional two dimensional (2D) monolayer cultures, the cellular function and

properties of 3D spheroids more faithfully replicate those of cells *in vivo* [3, 4].

The 3D cell culture system helps bridge the gap between 2D monolayer systems and animal models by providing an *in vitro* cell model system mimicking features of the *in vivo* environment, and thereby providing relevant answers for which animal models fails in spite of spending time and cost[5]. The uses of 3D cell culture systems can minimize the use of animal models for the translational research [6].

Multicellular spheroids are important 3D cell culture models which have been extensively used in drug screening tumor studies and tissue engineering [7]. So cells in spheroids have higher resistance to chemotherapy compared to cells grown in mono layers and this could be one reason why various chemotherapeutics lose their efficacy when they were tested *in vivo* [8].

The multicellular tumor spheroids have been a valuable model to provide more comprehensive assessment of tumor in response to therapeutic strategies [9]. Owing to their *in vivo* -like characteristics, three-dimensional (3D) multicellular tumor spheroid (MCTS) cultures are gaining increasing popularity as an *in vitro* model of tumors. A simple approach to the cultivation of these MCTS is the hanging-drop method. Cells are suspended in droplets of medium, where they develop into coherent 3D aggregates and are readily accessed for treatment [10, 11].

Colorectal cancer is among the highly metastatic and resistant solid tumors to chemotherapy and one of the most active agents currently being used revealed that 50% of patient's have responded to chemotherapy. However, development of new drugs required new strategies and methods to improve clinical outcomes [12].

In vitro cell cultures are important experimental tools in understanding the biology of cancer cells, as well as for the evaluation of potential therapeutic agents, and understanding of the mechanisms underlying their actions [13, 14].

Doxorubicin (Dox) is used for hematopoietic cancer and a wide range of solid tumors, including breast carcinoma, small-cell lung carcinoma and metastatic thyroid carcinoma [15, 16].

MATERIALS AND METHODS

Chemicals and drugs

Sulpho Rhodamine-B (SRB) and Doxorubicin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Culture media and growth supplements were purchased from Gibco / Life Technologies Co, (Carlsbad, CA, USA). Cell culture vessels were purchased from Nunc Co. (Roskilde, Denmark).

Cell culture

Human colorectal carcinoma (HCT 116) was obtained from VASERA (Giza, Egypt). Cells were maintained in RPMI media supplemented with 100 µg/mL streptomycin, 100 units/ml penicillin and 10% heat-inactivated fetal bovine serum in a

humidified, 5% (v/v) CO2 atmosphere at 37 ºC, the cells were sub-cultured tow times in week .

Cytotoxicity assessment of Doxorubicin against 2D culture of Human colorectal carcinoma (HCT 116 cell line)

The sulforhodamine B (SRB) method is used for cell cytotoxicity which depends on the measuring the cellular protein content. The assay has been optimized for the toxicity effects of compounds to adherent cells in microplates. Growing cells were collected using 0.25% Trypsin-EDTA and plated in 96-well plates at 2000 cells/well, the cell monolayers will treat with serial concentrations (0.01to100 µg) from doxorubicin. After 72 hrs. the cells will fix with 10% trichloroacetic acid for 1 hr. at +4C. The cells will wash with tap water 3 times and stain for 10 min. The excess of dye is removed by washing repeatedly with 1% acetic acid and leave the plate over night to dry. The cell protein dyed is dissolved in 10 mM Tris base PH 7:4 finally the plate was measure at 540 nm OD using ELIZA reader. The data are analysis using SigmaPlot version 12.0. [17, 18]

Cytotoxicity assessment of Doxorubicin against multicellular tumor spheroids (MCTS) of Human colorectal carcinoma (HCT 116 cell line)

Monolayers cells were collected from culture flasks using 0.25% Trypsin-EDTA and pelleted by centrifugation. Cells are then resuspended in growth medium at the density 40.000 cell per 20ul suspension and hanging drop were plated in flat shaped 96-well plate; Cells were incubated for 3 days to form multicellular tumor spheroids (MCTS) of HCT116 cell line. Multicellular tumor spheroids (MCTS) of HCT-116 were exposed to serial concentration (0.01to100 µg) of doxorubicin. After 72 hrs. treatment the cells were fixed by direct adding 20% trichloroacetic acid for 2 hr. at +4C. The cells will wash with tap water 3 times and stain for 30 min. The excess of dye is removed by washing repeatedly with 1% acetic acid and leave the plate over night to dry. The cell protein dyed is dissolved in 10 mM Tris base PH 7:4 finally the plate was measure at 540 nm OD using ELIZA reader. The data are analysis using SigmaPlot version 12.0 with minor modifications. [17, 18] [19],

RESULTS

Morphological changes of HCT116 cultured as monolayer (2D) and multicellular tumor spheroid (MCTs)

Morphological changes in HCT116 monolayer were obvious as treated cells started showing gradual cell shrinkage, cell rounding and detaching from the surface of tissue culture flasks, finally followed by cell swalling and rupture, on the other hand the proliferative changes of HCT116 MCTs indicated that induce the cell death in inner tumor spheroid region (necrotic zone) more than outer spheroid regions.

Cytotoxicity Profile of doxorubicin against HCT116 cells in monolayer (2D) and multicellular tumor spheroid (MCTs)

In order to examine the cellular responses in multicellular tumor spheroid (MCTS) and monolayer cultures we used doxorubicin at concentrations ranging from 0.0 µg to 100 µg to treat the HCT116 cells. The cytotoxicity parameters, IC50 and R-fraction were calculated using SigmaPlot software.

The IC50 of doxorubicin in the monolayers exposed for 72 h was 0.8µg with 8.43 R-fraction and 3.2 µg with 14.5 R-fraction in MCTS exposed for 72 h (Fig. 3&4). The previous results showed that the MCTS was more resistant to doxorubicin than the monolayers. The IC50 were drastically lower under monolayer conditions compared to MCS at all drug exposure time and culture conditions

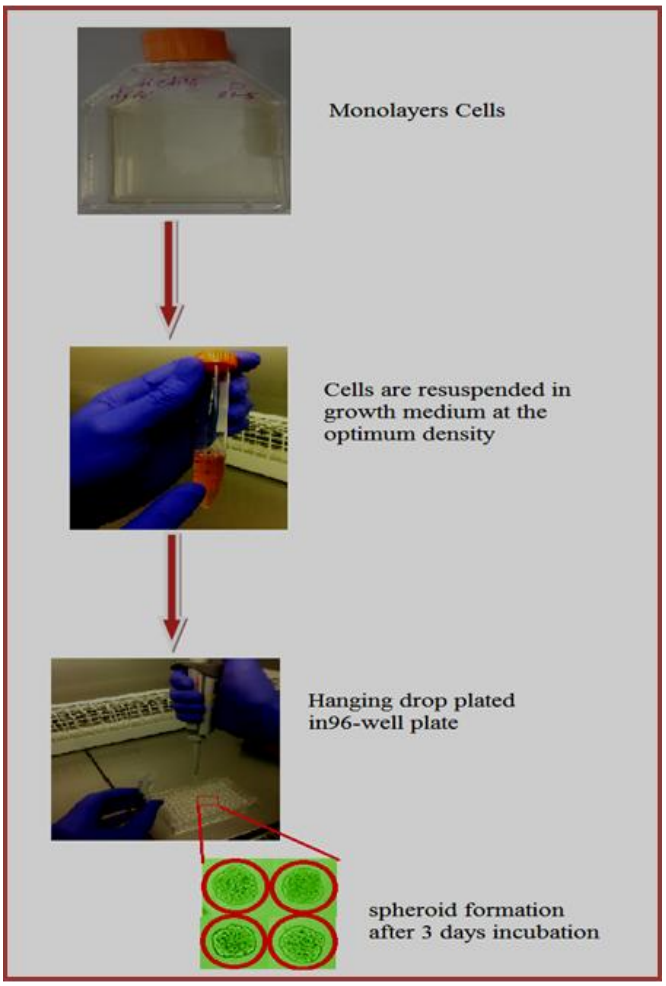

Fig.1. Preparation of multicellular tumor spheroids (MCTS) by the hanging-drop method. Monolayers are dissociated and pelleted by centrifugation. Cells are then resuspended in growth medium at the desired density, and 20µl suspension dispensed into each well of a 96-well plat. The plate then placed in an incubator for 3 days for spheroid formation.

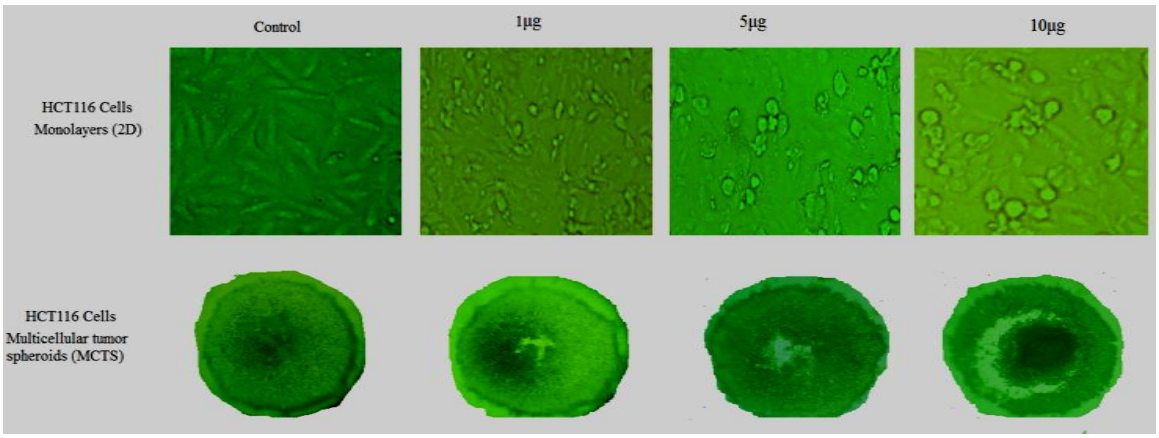

Fig.2. Phase-contrast images of human colon carcinoma HCT116 cultured as 2D monolayer and multicellular tumor spheroids (MCTS). Cells were treated various (1, 5 and 10 µg) concentrations of doxorubicin for 48hrs.

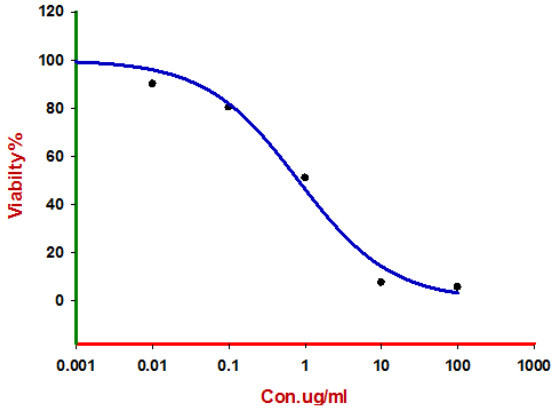

Fig (3) Dose response curves of doxorubicin against monolayer cultures of HCT116 cells by SRB assay. Cells were treated with various concentrations of doxorubicin for 72 hrs.

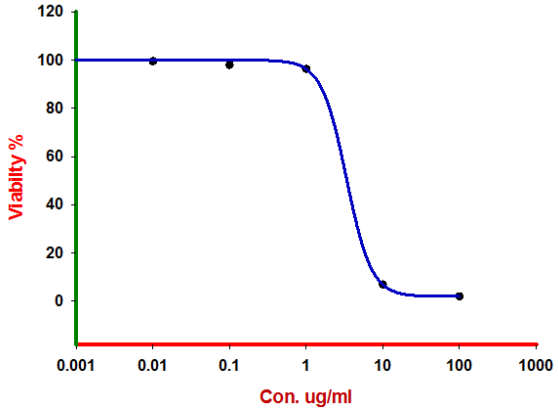

Fig (4) Dose response curves of doxorubicin against HCT116 MCTS by SRB assay. Cells were treated with various concentrations of doxorubicin for 72 hrs.

DISCUSSION

Most cell-based *in vitro* screening methods for new anti-cancer agents use cells grown on culture plates as monolayer in 2dimensional (2D) conditions. Most agents with significant antitumor activity using 2D culture in the screening process were found to be ineffective *in vivo* [20,21]. Tumor cells cultured in 3D may represent the biological behaviors of tumor cells *in vivo* better than 2D cells [22]. Moreover, this model could be used to study therapeutic problems related to metabolic and proliferative gradients [1].

A 3D culture environment is important to normal cell physiology. Cell-to-cell adhesion is a critical factor that drives the formation of 3D cellular spheroids. Although a few different systems have been used to grow 3D MSTC spheroids that in general display better differentiation capacities upon induction, most of them maximize the cell-to-cell contact to drive spheroid formation [2, 3].

The 3D *in vitro* culture system closely resembles *in vivo* tumor conditions; where cellular resistance and a penetration barrier to cytotoxic agents represent the major obstacles to obtain full efficacy[23]

In our previous study, we observed that the activity of doxorubicin has stronger anti-proliferation activity against HCT116 cell lines grown in 2D than 3D tumor spheroids whereas the IC50 value of 3D tumor spheroids HCT116 cells were about 4 times higher than those from 2D HCT116 cells.

The new drugs candidates undergo animal tests and clinical trials studies every year but almost all fails and only few gets through the Food and Drug Administration (FDA) patent approval after spending millions of dollars and 10-15 years of total drug discovery and development time [24]. 2D monolayer cell cultures fail to provide similar microenvironment and not give full cellular interactions. Developing 3D systems for cell growth provides a relevant and accurate response of extracellular matrix. They have great potential in improving cell based drug screening so as to mimic physiological cell-cell interaction to regulate proliferation and differentiation, which help in identifying toxic and ineffective substances at an earlier stage of drug discovery [25].

CONCLUSION

In the light of these data, our modified method not only confirmed the sensitivity variation between 2D and 3D systems but also showed the significance to improve and develop a new method to address and resolve the obstacle of 2D monolayer culture. This means and requires further studies on different types of cells in 2D and 3D culture systems.

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