

Cytotoxic Effects of Some Common Organic Solvents on MCF-7, RAW-264.7 and Human Umbilical Vein Endothelial Cells

Leila Jamalzadeh,¹ Hosein Ghafoori,^{1*} Reyhaneh Sariri,¹ Hanieh Rabuti,¹ Jila Nasirzade,¹ Hajar Hasani,¹ and Mahmoud Reza Aghamaali¹

¹Department of Biology, University of Guilan, Rasht, IR Iran

*Corresponding author: Hosein Ghafoori, Department of Biology, University of Guilan, Rasht, IR Iran. Tel/Fax: +98-133333647, E-mail: h.ghafoori@guilan.ac.ir

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Abstract

Background: Organic solvents are widely used in cell biology experiments. Despite increasing the solubility, they have some moderate toxic effects. Therefore, selecting the appropriate solvent along with the use of suitable concentration insures the accuracy and reliability of experimental results.

Objectives: The current study aimed to examine the cytotoxic effects of some organic solvents on various cell models including MCF-7, RAW-264.7 and human umbilical vein endothelial cells (HUVEC).

Materials and Methods: To evaluate the cytotoxicity effect of common organic solvents on the MCF-7, RAW-264.7 and HUVEC cells, multi-table tournament (MTT) colorimetric assay, the widely used and validated cytotoxicity test was applied. For this purpose, the selected cells were treated with different concentrations (0, 0.1%, 0.5%, 1%, 1.5%, 2%, 3% and 5% v/v) of four most commonly used organic solvents (acetone, ethanol, dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) and then subjected to MTT experiment.

Results: According to the obtained results, the cytotoxicity increased significantly with increasing the concentration of all four solvents compared to that of the control group. Studies with MCF-7, RAW-264.7 and HUVEC suggested that acetone, ethanol and DMSO at concentrations of 0.1% and 0.5%, had little or no toxicity, whereas higher concentrations inhibited the growth of all three cells. Compared with other three solvents, DMF displayed rather greater toxicity. Based on the results, proliferation of MCF-7, RAW-264.7 and HUVEC cells were inhibited by all used organic solvents, dose dependently.

Conclusions: Thus, the background experimental error can be reduced remarkably by maximal concentration of 0.5% ethanol, acetone and DMSO and 0.1% DMF in the final treatment medium.

Keywords: Cytotoxicity, MCF-7, RAW-264.7, HUVEC, Organic Solvents

1. Background

Organic solvents are often used as a vehicle to dissolve hydrophobic compounds in cell biology experiments (1-4). Besides strong solubility characteristics, they should be compatible with the culture medium without toxic effects on cells, since experiments with cell based systems are performed in growth medium (2, 3). Some of the commonly used organic solvents such as acetone (Log P, -0.24), ethanol (Log P, -0.31), dimethyl sulfoxide (DMSO) (Log P, -1.35) and dimethyl formamide (DMF) (Log P, -1.01) were selected in the current study. Based on reports from in vitro studies, possible toxic effects of these organic solvents are expected (5-7). Several authors have reported interference of frequently used solvents with cellular based assays (8, 9). Therefore, the solvent should be selected to optimize the solubility of poor water-soluble compound without adversely affecting the assay conducts, such as cell growth (10). Thus, the cell growth inhibition and cytotoxicity study of organic solvents are very essential and nec-

essary. A number of methods including colorimetric and fluorometric are currently used in the fields of toxicology and pharmacology to determine cellular viability following in vitro exposure to compounds (11). Each method has specific advantages, disadvantages and limitations and the suitable method is the users' choice (12). To select the appropriate solvent with suitable concentrations, the most standardized and validated test to estimate the cytotoxicity of compounds towards cells is MTT test. This assay is dependent on the ability of live but not dead cells to reduce significant amounts of water-soluble yellow MTT dye to insoluble violet formazan crystals, which can be measured by a colorimetric method (13). The technique is used in a number of laboratories and various modifications are introduced (14). Some studies have demonstrated that various cell lines show differences in their degree of sensitivity to the same solvent. Therefore, besides the appropriate solvent the other factor to be evaluated is the solubility power of solvent with respect to the specific type of cell line (6, 8).

It is hardly possible to identify a recent discovery that

has not used cell line models at some point during development, and cancer cell lines are the most popular ones (15). Cancer is one of the major causes of human death worldwide and the number of affected people is increasing. Breast cancer is one of the important causes of mortality in females (16) and in Iran ranks first among cancers diagnosed in females comprising 24.4% of all malignancies (17). By far, MCF-7 human breast cancer cell line is the most widely used breast cancer cell line (18), which is useful for in vitro breast cancer studies because it has retained several ideal characteristics particular to the mammary epithelium (16). The usefulness of the MCF-7 cell line as an investigative tool led to its widespread adoption in laboratories (19). Another model system selected to carry out this study was the RAW264.7 mouse macrophage cell line, which is extensively used as a reliable cell model for inflammation research (20). They are also used to study macrophage cellular physiology because of their ease of culture, rapid growth rate and phenotypic resemblance to primary macrophages (21). To date, a PubMed retrieval lists over 1500 publications that used the RAW264.7 cell line in the reported research work (22). The third selected cell model was human umbilical vein endothelial cell (HUVEC) line which is commonly used as a laboratory model system for various physiological and pathological processes, especially in angiogenesis research (23). It also provides a classic model system to study many aspects of endothelial functions and diseases (24). However, compared with other types of human endothelial cells, HUVEC offers several important advantages for in vitro studies. In particular, they are easier to harvest and maintain in culture and have a greater inherent and useful passage number in vitro. Therefore, the HUVEC model is valuable to investigate functions of human endothelial cells and their interactions with other cell types (25).

2. Objectives

Thus, the current paper studied the effects of common organic solvents with different concentrations towards the most popular cells: MCF-7, RAW-264.7 and HUVEC, which are cancer, macrophage and endothelial cells, respectively. On the other hand, in the context of cell culture-based testing of anti-proliferative property of compounds, little solvent evaluation is carried out so far. To the best of the authors' knowledge this is the first study to explore toxic effects of common solvents on widely used cell line models. Thus, the study could provide reference for optimizing solvent selection with regard to suitable concentrations.

3. Materials and Methods

3.1. Reagents

All culture media were obtained from Gibco (Invitrogen, Carlsbad, CA, USA) and 3-(4, 5 dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) was provided by Sigma-Aldrich, USA. All solvents, acetone, ethanol, dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) were of reagent grade and purchased from Merck, Germany. The purity of all chemicals used was in the range of 96% - 99.9%.

3.2. Cell Culture

MCF-7, RAW-264.7 and HUVEC cells were provided by the Iranian biological resource center (IBRCTM, Tehran, Iran) and grown in the Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All the cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ throughout the study and routinely grown in 25 cm² culture flasks and trypsinized to harvest after attaining confluence. Meanwhile, some differences were considered regarding trypsinization procedure for RAW-264.7 semi-adherent cells versus two other adherent cells. Following centrifugation (1300 g for 7 minutes), cells were resuspended in the culture medium and used for the following study.

3.3. Cytotoxicity Test-MTT Assay

To evaluate the cytotoxicity effect of acetone, ethanol, dimethyl sulfoxide (DMSO) and dimethyl formamide (DMF) on three different cell lines, viability tests were applied using MTT colorimetric assay. Briefly, all cell lines were seeded in 96-well plates at a density of 20×10^3 cells per well and then incubated at 37°C in 5% CO₂ to allow cell attachment. The medium was removed and replaced with fresh medium containing various concentrations (0, 0.1%, 0.5%, 1%, 2%, 3% and 5% v/v) of four organic solvents. After treatment for 24 hours, 10 μ L MTT (5 mg/mL) was added to each well and the plate was further incubated. Four hours later, all remaining supernatant were removed and 100 μ L of DMSO was added to each well to dissolve the resulting formazan crystals. Finally, absorbance was read at 570 nm using the enzyme-linked immunosorbent assay (ELISA) microplate reader (BioTek, USA) and the cell viability percentage was calculated using the equation: (mean OD of treated cells/mean OD of control cells) \times 100. The percentages of cell viability were used to determine the IC₅₀ values, which is the concentration of a chemical inhibiting 50% of the cell growth compared with that of the untreated control cultures.

3.4. Statistical Analysis

Each concentration was assayed in triplicates ($n = 3$) and repeated in two independent experiments. Statistical analyses were performed by SPSS statistical software (version 20.0, SPSS). Values with $P < 0.05$ were considered as statistically significant. Data were analyzed using one-way ANOVA followed by the Dunnett test and expressed as mean \pm SD.

4. Results

The cytotoxicity of four solvents towards MCF-7, RAW-264.7 and HUVEC cells was determined by MTT assay. Results are shown in [Figure 1](#). The data indicate that the rate of cell viability decreased significantly ($P < 0.05$) with increasing concentrations of all examined solvents compared to those of the control group and cytotoxicity increased dose dependently. In the current study, the absorbance of each experiment was similar among the three parallel experiments. Overall results showed that the inhibitory effect of solvents was similar with slight differences between the three cell lines. According to the results, dimethylformamide (DMF) displayed the highest cytotoxic effect among the solvents, followed by decreasing order-DMSO, ethanol and acetone. It was found that, among the selected solvents, acetone exhibited the least cytotoxicity against different cells. While the concentration of acetone was 0.1%, 0.5%, 1% and 1.5% (v/v), the corresponding cell viability rate observed in HUVEC cells were 98%, 95%, 94% and 92%, respectively. In addition, concerning MCF-7 and RAW-264.7 cells, the viability rate did not exceed $\sim 80\%$ at doses up to 1% and 1.5%, respectively ([Figure 1A](#)). Also, the effect of ethanol was very similar to that of acetone in the equivalent dose range and the IC_{50} values for both solvents were more than 5% for all of the three cells ([Figure 1B](#)). The cytotoxicity of DMSO was a little greater than those of acetone and ethanol. However, little cytotoxicity of DMSO towards cells was observed up to 0.1% with cell viability rate of more than 90%; whereas it dramatically increased at concentrations above 0.5% ([Figure 1C](#)). Interestingly, DMF presented a great cytotoxicity and descended rapidly at a very low concentration in such a way that RAW-264.7, MCF-7 and HUVEC cells at the highest concentration (5%), exhibited very low cell viability rate, about 11%, 12% and 16%, respectively. Furthermore, viability rates of acetone, ethanol and DMSO up to 0.5% were more than $\sim 80\%$; whereas DMF at equivalent concentrations showed great cytotoxicity, approximately less than 70%. Therefore, DMF displayed the most cytotoxicity towards all different cells ([Figure 1D](#)).

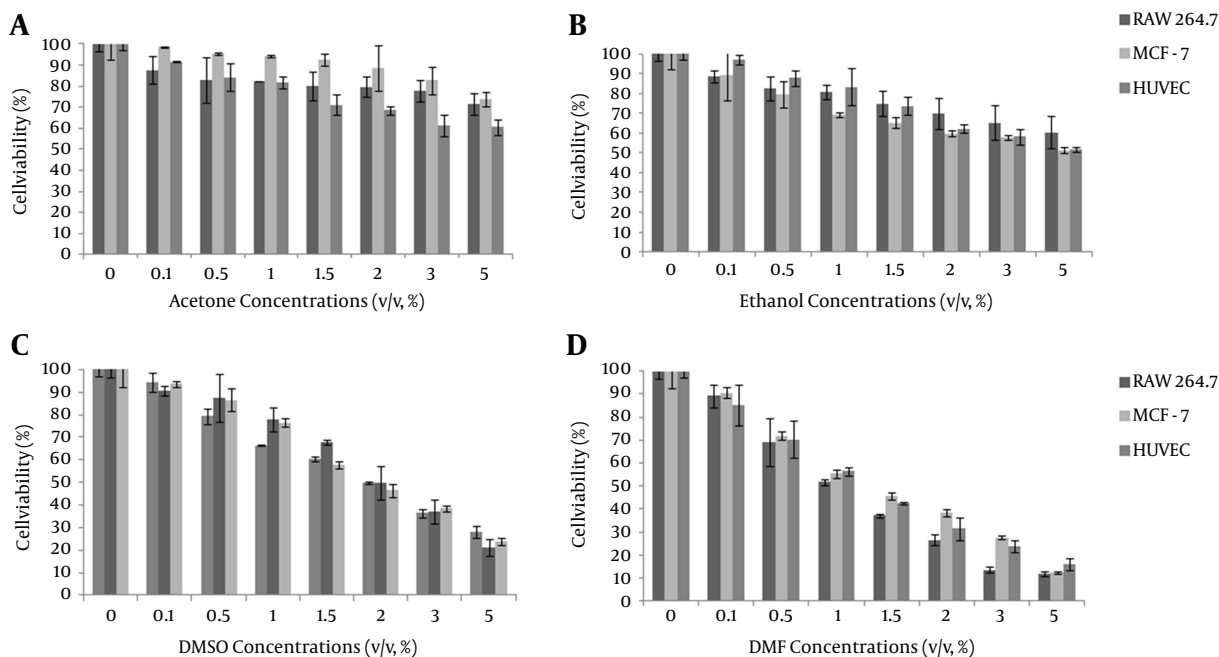
Accordingly, there was no obvious difference in IC_{50} value of these solvents between RAW-264.7, MCF-7 and HUVEC cells. In all cells, IC_{50} values of DMSO and DMF were

1.8% - 1.9% (v/v) and 1.1% - 1.2% (v/v), respectively. Also, in the case of acetone and ethanol, the calculated IC_{50} values were more than the examined concentrations i.e, 5% (v/v). Obviously, among the solvents used, DMF exerted the maximum inhibitory effect on cells. Therefore, it seems that acetone, ethanol and even DMSO could be solvents of choice acceptable to be used at concentrations $< 0.5\%$ (v/v) towards the examined cells and possibly for other cell lines.

5. Discussion

Cell culture systems including cell lines of human or murine origin are extensively used to study the effects of pharmacological relevant compounds and natural products (3). Since many of these compounds are water-insoluble, the use of organic solvent is necessary (4). However, a common concern when introducing organic solvents to the growth medium is toxic effects of the solvent on cells (2, 5, 8, 9). In addition, using unsuitable solvents might cause the loss of biological activities in compounds; therefore, it may seriously affect the outcome of the experiment (1, 3). Thus, the current paper investigated the cytotoxic effects of four commonly used organic solvents against three different cell culture systems: MCF-7, RAW-264.7 and HUVEC cell lines. The study aimed to select suitable solvent to dissolve hydrophobic compounds. The selected solvents should have the advantages of high solubility power, compatibility with the media and no adverse impact on cells. For this purpose, the cytotoxicity of acetone, ethanol, DMSO and DMF against MCF-7, RAW-264.7 and HUVEC cell lines was evaluated by the widely used MTT assay. Results indicated that all the solvents exerted cytotoxic action dose dependently; since the cell viability percentage of the three different cell lines decreased significantly by increasing the concentration of solvents compared to that of the control. However, little differences were observed between the three cell lines. Therefore, it seems that acetone, ethanol and DMSO might be more compatible solvent vehicles toward all three cells, respectively; while DMF is the worst owing to its greater cytotoxicity. In addition, the effects of ethanol and acetone were nearly similar with slight differences between the cells.

Interestingly, the obtained results were consistent with the Q3C solvent classification. According to Q3C guideline, solvents are divided into four classes (1-4). Acetone, ethanol and DMSO were placed in the safest category, class 3 solvents. Solvents in class 3 may be regarded as less toxic with lower risk to human health. While, DMF was placed in class 2 solvents. The use of solvents in class 2 should be limited in pharmaceutical products because of their inherent toxicity (26). Acetone is employed as a solvent for compounds used to study the appearance of

Figure 1. Cell Viability Percentage of MCF-7, RAW-264.7, and HUVEC Cells Exposed to Different Solvents

A, Acetone; B, Ethanol; C, DMSO; D, DMF for 24 hours calculated from the absorbance values obtained from the MMT assay. The cell viability percentage was the mean absorbance of solvents group at different concentrations (0, 0.1, 0.5, 1, 1.5, 2, 3, and 5% v/v) divided by that of corresponding control group. The bars represent the mean SD obtained in three independent experiments.

malignancy in tissue cells. Although, little reports could be found in the literature concerning the effects of acetone upon cells growth in vitro, it is demonstrated that acetone changes the permeability of the cell membranes (27). The current study results clearly indicated that acetone was a non-toxic solvent since it could not inhibit the growth of the studied cells by 50% at examined ranges from 0.1% to 5% (v/v). Thus, it seems to be the most suitable solvent to dissolve the hydrophobic compounds in such cells. Ethanol is also used as a solvent for hydrophobic compounds in experimental studies (28). Previous studies demonstrated that ethanol modulates the cell growth and suppresses cell proliferation dose dependently (29). It is also evident that ethanol regulates apoptosis or necrosis by generating reactive oxygen species (ROS) or inducing transient arrest of cell division (30). Although many reports describe the cellular effects of ethanol at high concentrations, only a few relate its effects at low concentrations (31). In the present study, ethanol exhibited somewhat less cytotoxicity towards cells following acetone and the IC_{50} values for each cell were higher than 5% (v/v). This reflects its low cytotoxicity as well as high safety on the examined cells. Dimethyl sulfoxide (DMSO) is a small amphiphilic molecule, which is widely employed in cell bi-

ology as a cryoprotectant, hydroxyl radical scavenger, cell fusogen and more importantly as an effective penetration enhancer; since it is known to enhance cell membrane permeability of compounds (32, 33). It also is frequently used as an efficient solvent in biological studies to dissolve hydrophobic compounds and as a vehicle for compound therapy (34). This characteristic, in addition to its low toxicity, has led to its ubiquitous use and widespread application (35). A great number of toxicological and medical studies are performed with DMSO to determine the safety of this chemical (10, 27). Although clinically beneficial in some situations, DMSO can have some adverse side effects. It is reported that DMSO could cause severe cell damage through interacting with the metabolism and membrane of cells (36). However, little is known about its potential cytotoxic side effects on different cell lines. In addition, the effects of DMSO on cellular function are studied in a large number of cell types with variable results (34-37). Several studies indicated that proliferation of various cells was obviously inhibited by DMSO at different concentrations (38). The current study suggested that the cytotoxicity of DMSO was only little greater than that of acetone and ethanol, especially at very low doses i.e., < 0.1%, wherein DMSO exhibited the least toxicity among other solvents. However,

the cytotoxicity was greater with increasing the concentrations. Also, according to the current study results, the IC_{50} values of DMSO on three diverse cells were very similar and about 1.1% - 1.2% (v/v) for all cells. Finally, DMF is a type of polar solvent which causes morphological changes in the cells as well as alterations in their growth properties (39, 40). It is also a potent differentiation inducing agent with growth inhibitory activity in tumor cells, yet its effect on some cancer cells remains unclear (40, 41). The obtained data obviously revealed that, compared to other three solvents, DMF exhibited the highest cytotoxicity and inhibited the proliferation of cells most effectively, while acetone and ethanol did not demonstrate such prominent effects on cellular growth. Since cell based assays should be validated based on cytotoxic effects of solvents used, the current study highlighted safety concerns of using some preferred organic solvents in biological assays. Some researchers recommended that to solubilize compounds, it is better to compute absolute solvent final concentrations and include an untreated control group in addition to solvent vehicle control to check for its toxicity (10). Also, some others emphasize on the need to lower the concentrations of solvents in toxicology tests as far as possible (3, 42).

5.1. Conclusions

Acetone was the most favorable solvent to dissolve the compound in cell growth in vitro and is a non-toxic solvent, since it demonstrated the least growth inhibitory effects on cells with an average of 85% cell viability in volume (0.1% - 1% v/v). Therefore, it could be concluded that acetone, ethanol and even DMSO, at concentrations < 0.5% (v/v) might be compatible solvent vehicles towards the examined cells.

Finally, a simple approach is presented to select a suitable solubilizing agent that would enable testing the anti-proliferative activity of a hydrophobic compound according to the cell type. The current study observations are important when selecting an appropriate solvent in cell based studies for MCF-7, Raw-264.7 and HUVEC cell lines.

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Footnotes

Authors' Contribution: Hosein Ghafoori and Reyhaneh Sariri: study concept and design; Jila Nasirzade: acquisition of data; Leila Jamalzadeh and Hosein Ghafoori: analysis and interpretation of data; Leila Jamalzadeh: drafting

of the manuscript; Mahmoud Reza Aghamaali: statistical analysis; Hanieh Rabuti: study supervision.

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