ASM Sc. J., 17, 2022

https://doi.org/10.32802/asmscj.2022.1238

Determining the Population Doubling Time of HepG2 and Huh-7 Cells and the Toxic Effect of Dimethyl Sulfoxide (DMSO)

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Developing cell lines that carry promising qualities closest to human hepatocytes in drug studies is a dream of many research laboratories. About 90% of drugs are metabolised by the liver. Therefore, most drug discovery studies utilise hepatocytes to understand the basic mechanism of the drug's breakdown. To assure hepatocytes survival, various solvents were tested for their cytotoxicity on the cell line. Since most drugs are weakly soluble in water, they can be dissolved in an aprotic solvent. To obtain accurate results, the requirements of these solvents are biocompatible and non-toxic to the cells. One of the most commonly used solvents is dimethyl sulfoxide (DMSO). This study aims to understand the cell characteristic of cells doubling time and investigate the toxic effect of DMSO using the cell proliferation measurement technique. The toxicity was measured on liver-derived cell lines HepG2 and Huh-7. Cell growth and morphology were observed with an inverted phase microscope while cell viability was counted using Vi-Call XR 2.03. Results showed that a doubling time of 32 hours would be best for treatments on both cells. A concentration of more than 0.40% DMSO has significant toxicity and caused inhibition of proliferation on both cell lines, resulting in low cell count and higher cell death. Ethanol and methanol were observed as good solvents since they have low toxicity. However, drugs that were only dissolved in a low concentration of DMSO (0.05-0.2%) gave the best result without any harmful effect on cell proliferation.

Keywords: DMSO concentration; Hepatocytes; HepG2; Huh7; toxic concentration; cell doubling

I. INTRODUCTION

The hepatic cell line is widely used in *in vitro* studies as these cell lines provide a stable and unlimited source of hepatocytes-like cells. The most well-known human hepatoma cell line is the HepG2 cells and Huh-7. Hep-G2 derived in 1975 from a 15 year old Caucasian male from Argentina with a well-differentiated hepatocellular carcinoma that was immortalised, has been widely used for various hepatotoxicity examinations (Aden *et al.*, 1979). Another widely available cell line was established in 1982 from a Japanese patient with a well-differentiated hepatocellular carcinoma (Nakabayashi *et al.*, 1984). Huh-7, a liver carcinoma cell from the Japanese Cell Research Bank also known as JTC-39; Japanese Tissue Culture-39 have been used in drugs toxicity studies as this cell maintains key hepatic function, hepatic gene regulation, and hepatic disease model (Nakabayashi *et. al.*, 1984; Dorr *et. al.*, 2017; Hwang *et al.*, 2006). Developing cell lines that carry promising qualities as close as to human hepatocytes in induction studies is a dream of many research laboratories. A cell line that would potentially be used to investigate drug metabolism and a near-perfect substitute for human hepatocytes in induction studies must express the necessary transcription factors, drug-metabolising enzymes, and transporter protein (Kumari *et. al.*; 2020, Dai *et al.*, 2017).

In cell line-based drug metabolism studies, the organic solvent agent is an important component used to dilute test drugs, especially drugs that are weekly soluble in water. One of the most widely used organic solvents is dimethyl

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sulfoxide (DMSO). DMSO is used to dissolve poor water-soluble compounds due to its high boiling point (189°C), relatively high freezing point (18.5°C), and its miscible nature with water and a wide range of other organic solvents. The significant properties of DMSO are colourless, polar, and also that it dissolves in polar and nonpolar compounds (Singh *et al.*, 2017). Evaluation and determination of the maximum concentration of DMSO that could be used for dissolving the drugs in the biological assay are important as they could lead to misleading or wrong interpretation of induction assay outcome. If the solvents used in a study are toxic to the cells over treatments, the effect of the drugs will be wrongly evaluated (Nguyen *et al.*, 2020). This study aimed to understand the cell characteristic of cells doubling time and investigate the toxic effect of DMSO for HepG2 and Huh-7 cells.

II. MATERIALS AND METHODS

Dulbecco's modified Eagle's medium (DMEM) was purchased from Simply Biologics, (GeneDirect, Taiwan). Fetal bovine serum (FBS), Acutase, and Penicillin-Streptomycin were purchased from Gibco (Pittsburgh, PA, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich® Solution (Darmstadt, Germany), 24-wells/plates.

A. Cell Lines and Cell Culture

Huh-7 (EP-CL-0120) was purchased from Elabscience Biotechnology Inc and HepG2 was purchased from ATCC. Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose and pyruvate supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100 U/ml streptomycin, referred to as "media" throughout this study. Cells were dissociated by Acutase and seeded into a 24-well/plate with 50 000 cells/well that were used through over study as starting the number of live cells.

B. Doubling Time Assay

24-well plates were used for the main experiment in which cells were seeded in six (6) groups, each were incubated at 2,4,8,16,32 and 64 hours, respectively. All cultures were tested in quadruplicates at 37°C with 5% CO_2.

C. DMSO Treatment Experiment

The cells were seeded in 24 well plates at 50 000 cells/well. Cultures were treated with 0.05, 0.10, 0.20, 0.40 and 0.80 % of the DMSO (v/v) in 0.5 ml of DMEM media with 10% FBS and a control of only 0.5 mL of DMEM media without any DMSO. Treated cells were cultured in quadruplicates at 37°C with 5% CO_2. Data were collected after five days of incubation.

D. Cell Viability Assay

Cell growth and morphology in the respective test were captured with an inverted phase microscope. The cells were counted using Vi-Call XR 2.03, Beckman Coulter, Inc (Life Sciences, United State)

E. Statistical Analysis

The data were analysed with GraphPad Prism 9.2.0, as the mean, standard deviation, Pearson correlation, and two-way ANOVA for multiple comparisons of values in each category were calculated.

III. RESULTS AND DISCUSSION

A. Comparison of Doubling Time HepG2 and Huh-7

In this study, cell doubling time analysis was carried out to determine the characteristics of parents' cells of Huh-7 and HepG2. Figure 1 represents the average number of cells at 2,4,16, 32, and 64 hours for HepG2 and Huh-7 cells. Referring to the histogram below, HepG2 achieved its doubling time at 16 hours as opposed to Huh-7, that took over 64 hours.

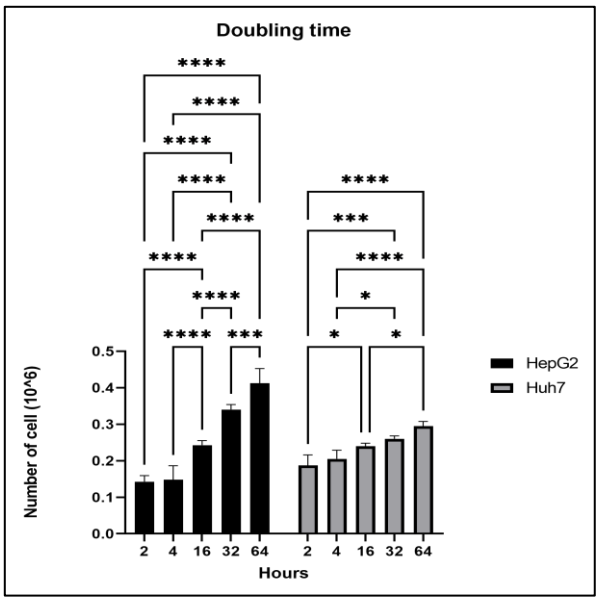

Figure 1. The average of cell viability at 2,4,16, 32 and 64 hours of HepG2 and Huh-7. Error bar represents standard deviation. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05

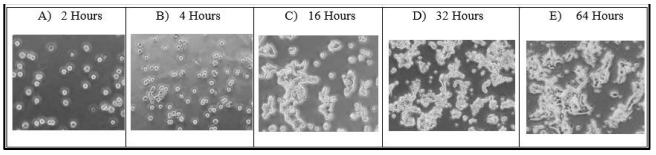

Figure 2. The wells seeded with HepG2 cells were observed at different doubling times; (A) 2 Hours, (B) 4 Hours, (C) 16 Hours, (D) 32 Hours, and (E) 64 Hours. The result is shown at x400 magnificent power.

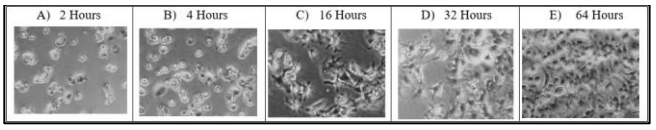

Figure 3. The wells seeded with Huh-7 cells were observed at different doubling times (A) 2 Hours, (B) 4 Hours, (C) 16 Hours, (D) 32 Hours, and (E) 64 Hours. The result is shown at x400 magnificent power.

Based on Figure 2, Huh-7 did not show significant growth between 2 hours and 4 hours, but it showed a significant difference after 32 hours. Huh-7 doubled its population in twice the time it took for HepG2 cells to double in amount. Referring to Figure 2 and Figure 3, the cell numbers increased rapidly in the initial phase. This is followed by the differentiation phase of the cells. This observation differs from a previous study that observed HepG2 cells doubled over 48 hours after seeding (Norouzzadeh, 2016). The doubling time at 16 hours for HepG2 culture for this study was consistent with other studies that have demonstrated a shorter population doubling time of hepatocytes cells (Wen-Sheng, 2003).

B. Comparison of the Toxic Effect of DMSO on HepG2 and Huh-7

A comparison was made to evaluate the toxicity effect of DMSO solvent on HepG2 and Huh-7 cell growth and proliferation in *In vitro* study. To do so, the tested cells were exposed to different concentrations of DMSO and evaluated for their growth performance over the five days.

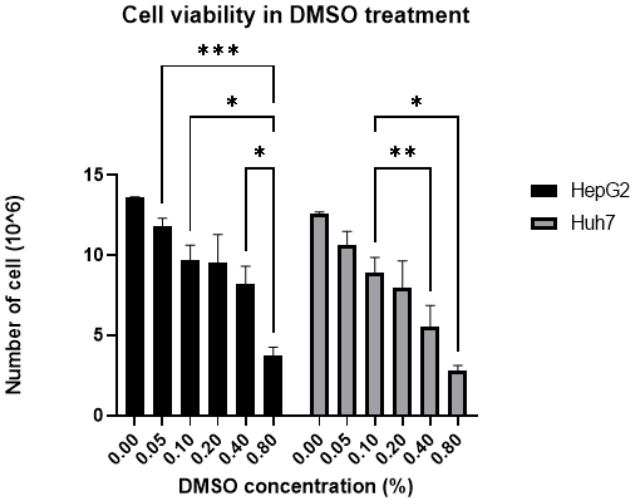

Figure 4. The average of cell viability at 0.00, 0.05, 0.10, 0.20, 0.40, 0.80 % of DMSO concentration for HepG2 and Huh-7. Error bar represents standard deviation. ***p<0.001, **p<0.01, *p<0.05

Figure 4 showed a downtrend pattern as the concentration of DMSO increased. DMSO exhibits its toxicity to the cells after five days of exposure, as the percentage of DMSO is increased, the cell viability was observed to be decreased which indicates that these cells were expired. The minimum concentration of DMSO that caused toxicity to the cells was above 0.4%. DMSO concentrations at 0.05%, 0.10 % and 0.20% did not show any significant alteration in cell viability of HepG2 and Huh-7 when compared with other concentrations. This proves that 0.20 % of DMSO concentration is the safest amount to not significantly

reduce the viability of cells. The number of counted live cells found were inversely correlated with the increasing DMSO concentration from 0.05-0.80% (Figure 3). This observation might be associated with cell cycle arrest, cell differentiation, or cell death as stated in the previous study (Singh *et al.*, 2017). Figure 4 showed that at less than 0.10% of DMSO, the live cell count for HepG2 and Huh-7 were found to increase as the cells were seeded at 50 000 cells/well on a 24-well plate. Supporting these findings by other studies showed that a low concentration of DMSO may have potential effects on cell viability and proliferation (Singh *et. al.,* 2017; Nguyen *et. al.,* 2020; Verheijen, 2019; de Abreu Costa *et al.,* 2017).

B. Correlation of Doubling Time and DMSO Toxicity between HepG2 and Huh-7

This experiment was carried out on a 24-well plate. Figure 5 shows the doubling time of HepG2 and Huh-7. There is a positive correlation with the increasing culture incubation time. At 32 hours of incubation, graph 5(D) shows the highest number in positive correlation (r = 0.7949). This suggests that 32 hours represents the strongest correlation doubling time for both cells.

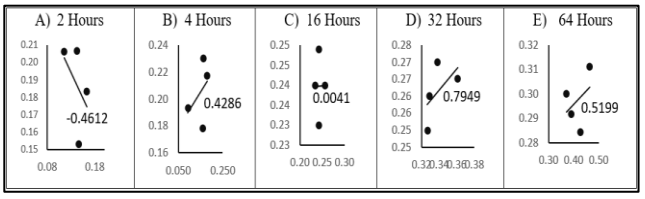

Figure 5. Correlation of doubling time between HepG2 and Huh-7 (A) 2 Hours, (B) 4 Hours, (C) 16 Hours, (D) 32 Hours (E) 64 Hours. Results were calculated using Pearson correlation.

A high dose of DMSO was initially investigated for medical application purposes, nowadays, a lower dose of DMSO is commonly used as a solvent and for cryopreservation reagent in cell culture studies. In the correlation analysis of five different doses of DMSO toxicity between HepG2 and Huh-7, the live-cell count was shown to have the highest positive correlation at 0.2% of DMSO concentration (Figure 6). However, this analysis is only considering DMSO concentration. Another variable that might affect cell proliferation is cell exposure time. A study found that exposure time to DMSO can be more harmful than the concentration parameter itself. Cells exposed to 10% of DMSO for one hour did not affect the viability of the cells in contrast to 0.2% DMSO was reported to increase cell death after seven days (Kloverpris *et al.*, 2010).

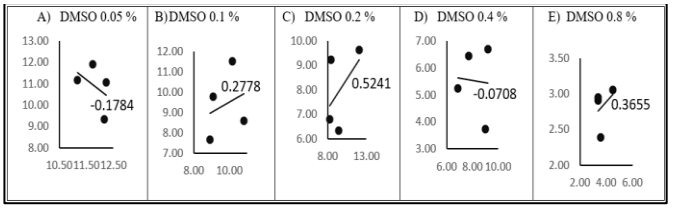

Figure 6. Correlation of DMSO toxicity between HepG2 and Huh-7; (A) DMSO 0.05 %, (B) DMSO 0.1 %, (C) DMSO 0.2 %, (D) DMSO 0.4 %, and (E) DMSO 0.8 %. Results were calculated using Pearson correlation.

IV. CONCLUSION

This study investigates the best doubling time and DMSO dose to be used in any subsequent experiments. In case of both cells, 32 hours would be the best time to be induced for any experimental treatment on both cells. In term of the mechanism of toxicity, it has been suggested that DMSO will affect the plasma membrane's physical properties by the interaction of DMSO with the plasma membrane, allowing the formation of the pores that contributes to the decreasing in membrane selectivity and increasing the cell's permeability (de Abreu Costa *et al.*, 2017). Referring to figures 4 and 6, in any long-term study that may involve drug treatment using HepG2 and Huh-7 in the future, 0.1% of DMSO concentration was observed to be the safest concentration. This was also in concordance with a previous study reporting a low concentration of DMSO of between 0.05-0.20% may lead to the stimulation of cell proliferation for some cell types such as RPMI-8226 myeloma cells (Wen *et al.,* 2015). Further investigations are needed to validate the DMSO toxicity effect on the molecular level as it is essential to keep DMSO concentration at the most effective in HepG2 and Huh-7 hepatocytes cell lines.

V. ACKNOWLEDGEMENT

Authors would like to acknowledge the Fundamental Research Grant Scheme (project code: 600-IRMI/FRGS5/3/365/2019) from the Malaysian Ministry of Higher Education for funding this study.

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