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Research Article

A STUDY OF POTENTIAL CYTOTOXIC EFFECT OF 1,4 DIOXANE ON HUMAN HEPATIC CELL LINE (HEP10)

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ABSTRACT

1, 4 Dioxane is a highly toxic inflammable substance present in contaminated air, tap water and a large variety of products ranging from shampoos, moisturizers to detergents, toothpaste and cosmetics. Canada and Qatar have even banned cosmetics and shampoos that contain 1, 4 Dioxane. This study aimed to investigate the potential cytotoxic effects of 1, 4 Dioxane on human hepatic cell line (HEP10) following exposure to different concentrations; which are 500 ppm, 2000 ppm, 5000 ppm and 10,000 ppm w3wwfor four different durations of time; 1, 7, 14 and 21 days . Cell viability and apoptosis level was assessed for all plates using comparison between MTT Assay and LDH Assay. Lower concentration of 1,4 dioxane has showed relatively high cell toxicity for LDH Assay which wasn't witnessed in previous studies. MTT Assay's reliability is questionable for checking cell viability.

INTRODUCTION

1, 4 Dioxane is a highly inflammable, colourless liquid completely miscible in water. (Mohr, Stickney & Diguiseppi, 2010).It is used in many products, including paint strippers, dyes, greases, varnishes and waxes. 1, 4-Dioxane is also found as an impurity in antifreeze and consumer products such as deodorants, shampoos and cosmetics. These products such as soaps, shampoos, toothpaste contain sodium laureth sulfate (SLES) an anionic detergent and surfactant; contain traces up to 279 parts per million (ppm) of 1, 4-dioxane; this is formed as a by-product during the ethoxylation step of its synthesis. (Kosswig, 2000)

It is used as a purifying agent in the manufacture of pharmaceuticals and is a by-product in the manufacture of polyethylene terephthalate (PET) plastic (EPA, 2014). Traces of 1,4-dioxane may be present in some food supplements, food containing residues from packaging adhesives or on food crops treated with pesticides that contain 1,4-dioxane as a solvent or inert ingredient (EPA, 2014).

1,4-Dioxane is also used as a solvent for cellulose acetate, ethyl cellulose, benzyl cellulose, resins, oils, waxes, some dyes, and other organic and inorganic compounds (EPA,2000). Table 1 given below represents the amount of 1,4 dioxane in ppm present in various products.

Table 1 Concentration of 1,4 Dioxane in various products

Products	Concentration (ppm)
Food Additives	10
Dietary supplements-Spermicide	10
Detergents – Conventional	55
Detergents- Natural	6
Shampoo	300
Household Products	160
Dish washing products	65
Moisturizers	4
Bath foam	41
Body Gel	16
Hair lotion	108
Soap	7
Cosmetics	4
Baby lotion	11

Mechanism of Action

1,4 Dioxane can be exposed to by breathing contaminated air, tap water and consumer products (Atsdr.cdc.gov, 2012). Figure 1 depicts the mechanism of action of 1,4 Dioxane through three different modes such as through inhalation, dermal and oral.

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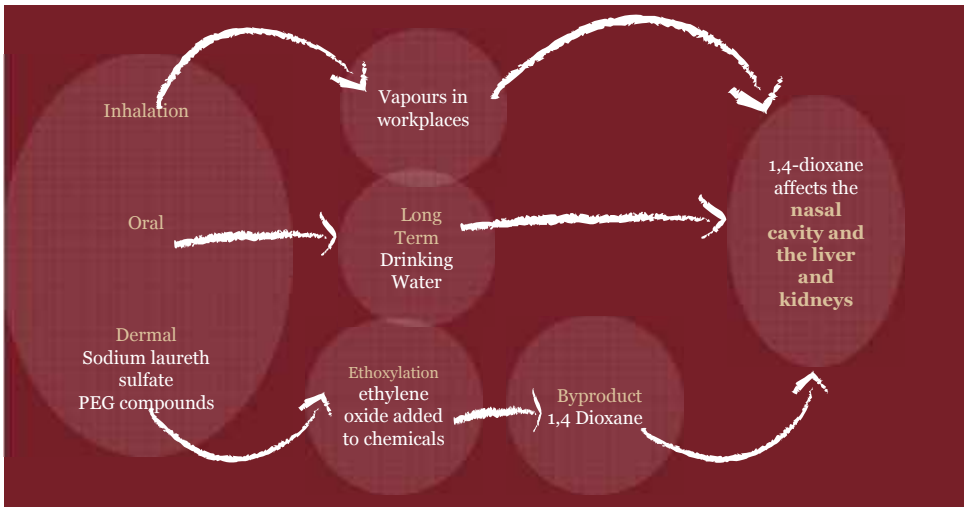

Figure 1 Mechanism of Action of 1,4 Dioxane.

Toxicology Report of 1,4 Dioxane
Acute toxicity (Sigmaaldrich.com, 2016)

- LD50 Oral - Rat - 4.200 mg/kg
- LC50 Inhalation - Rat - 2 h - 46.000 mg/m3
- LD50 Dermal - Rabbit - 7.858 mg/kg

Regulations for 1,4 Dioxane

1. The EPA has determined that exposure to 1,4-dioxane in drinking water at concentrations of 4 mg/L for one day or 0.4 mg/L for 10 days is not expected to cause any adverse effects in a child. (Atsdr.cdc.gov, 2012).
2. OSHA set a legal limit of 100 ppm 1,4-dioxane in air averaged over an 8-hour work day.
3. The National Academy of Sciences (NAS) established a maximum specification of 10 ppm for 1,4-dioxane in the ingredient polysorbate, a food additive.
4. FDA considered 10 ppm to be an acceptable limit for 1,4-dioxane during its evaluation of a spermicide, N-9, in a contraceptive sponge product.
5. FDA also set a limit on 1,4-dioxane at 10 ppm in approving glycerides and polyglycerides for use as excipients in products such as dietary supplements.
6. FDA keeps a record of raw materials and products contaminated with 1,4-dioxane. (Atsdr.cdc.gov, 2012).
7. Health Effects of 1,4 Dioxane

Limited information exists regarding the health effects of 1,4-dioxane oin humans. Yet, the available data are sufficient to clearly identify the liver and kidneys as the target organs for 1,4-dioxane toxicity following short-term exposure to relatively high amounts of 1,4-dioxane, regardless of the route of exposure. (Atsdr.cdc.gov, 2012)

Liver effects have occurred in humans and animals exposed to 1,4-dioxane, and the data in animals suggest that they occur regardless of the route of exposure.

Case Study 1

An occupational study and a case report provided a detailed description of the liver pathology in subjects following exposure to 1,4-dioxane that resulted in deaths within 1–2 weeks after the exposure.

Upon post-mortem examination, enlarged and pale liver and centrilobular necrosis were commonly observed. (Atsdr.cdc.gov, 2012)

Case Study 2

One study provided detailed descriptions of liver pathology in several animal species exposed intermittently to 1,4-dioxane by inhalation for a period of up to 13 weeks and also exposed orally and by dermal contact. Both lethal and non-lethal concentrations (1,000–10,000 ppm) caused degrees of degeneration that varied from cloudy swelling to large areas of complete necrosis. (Atsdr.cdc.gov, 2012)

Case Study 3

There is a report of 5 persons exposed to 1,4-dioxane in a synthetic silk factory in England who became ill with symptoms of renal insufficiency and died within 5 to 8 days. The affected persons had worked in a process which had been used in the factory for a long time without any adverse effects on health. One to two weeks before the persons became ill, one of the two machines was readjusted for technical reasons and this resulted in an increase in the workplace 1,4-dioxane concentration. At the same time, the shift length was increased from 8 to 12 hours. All the persons who died had worked at the readjusted machine. Autopsy revealed swollen kidneys subcapsular bleeding and pale, enlarged livers. Microscopic examination of the kidneys revealed haemorrhage in and around the glomerulus, and cortical necrosis. In addition, central lobular necrosis was detected in the liver. (1,4-Dioxane [MAK Value Documentation, 2003], 2012)
Controversy with 1, 4 Dioxane

Canada and Qatar has banned cosmetics and shampoo due to the presence of 1, 4 Dioxane respectively. The Canadian government regularly updates a Cosmetic Ingredient Hotlist that includes hundreds of chemicals and contaminants prohibited and restricted from use in cosmetics such as formaldehyde, triclosan, selenium, nitrosamines and 1,4-dioxane. (Figure 2) (Safe Cosmetics, 2008)

Qatar's expert panel which issued the order said the compound 1,4 dioxane was found above 10 parts per million (ppm) in the

two variants of the shampoo brand. The compound in excess could cause cancer and other skin diseases.

Qatari authorities said the decision to recall the two Head & Shoulders variants (Lively & Silky and Shine) must be strictly followed by all shops. “The ban is still in place”, Dr Mohammed Saif Al Kuwari, head of labs at the Ministry of Environment, said in a text message sent to The Peninsula. (Toumi, 2010)

The current Our research aimedis to evaluate the cytotoxic effect of 1,4 Dioxane on human hepatic cell line (Hep10) atfor different concentrations and for different durations of time.

METHODOLOGY

Research Design

HEP10 cells were grown using HEP10 culture media according to the manufacturer protocol. For morphological examination, pictures of cells were taken at day 0 at regular intervals while culturing the cells and after adding the drug as well. Cultured Hep10 Cells were exposed to four different concentrations; 500ppm, 2000ppm, 5000ppm and 10,000ppm and each concentration plate was exposed for four different durations of time;. Different concentrations include 500ppm, 2000ppm, 5000ppm and 10,000ppm. Each of these plates was exposed for 1, 7, 14 and 21 days to observe the possible cytotoxic effect over thesea periods of time. Positive and Negative control plates for each concentration were also cultured. Each cultured plate went through two different kinds of assay to check cell viability and apoptosis.

The two assays performed are – MTT Cell Proliferation Assay & LDH Cytotoxicity Assay.
LDH Cytotoxicity Assay

CytoTox 96 Non-Radioactive Cytotoxicity Assay is a colorimetric alternative to radioactive cytotoxicity assays. The CytoTox 96 Assay quantitatively measures Lactate dehydrogenase (LDH). LDH is a soluble cytoplasmic enzyme that is present in almost all cells, it’s released into extracellular space when the plasma membrane is damaged, it’s useful for monitoring cell death and disruption, it’s an oxidoreductase present in a wide variety of organisms.

To detect the leakage of LDH into cell culture medium, a tetrazolium salt (INT) is used in this assay. LDH catalyses the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD[+]. In the first step, LDH produces reduced NADH when it catalyzes the oxidation of lactate to pyruvate. In the second step, a tetrazolium salt is converted to a colored formazan product using newly synthesized NADH in the presence of an electron acceptor (Nachlas, M. *et al.*1960). The amount of formazan product can be colorimetrically quantified by standard spectroscopy. Because of the linearity of the assay, it can be used to count the percentage of necrotic cells in a sample. Since LDH is a fairly stable enzyme, it has been widely used to evaluate the presence of damage and toxicity of tissue and cells. Quantification of LDH has broad range of applications.

LDH Cytotoxic Assay was performed according to the following Pprotocol: (Decker, T. & Lohmann-Matthes *et al*. 1988)

1. The cells weare seeded the day before analysis at 2 x 104 cells in 100 µl per well in 96-well plates.
2. After induction of cell death, 50 µl of cell supernatant iwas placed in a 96-well plate.
3. Followed bying addition of 50 µl of reconstituted substrate mixture and incubation for 30 min at room temperature.
4. 50 µl of stop solution wais added to each well and optical density was measured at 492 nm is measured.
5. Results weare expressed as optical absorbance values.

MTT Assay was performed according to the following protocol

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) assay is a colorimetric assay for assessing cell viability. It is based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity. Since for most cell populations the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the in vitro cytotoxic effects of drugs on cell lines or primary patient cells (Sittampalam, G *et al*., 2004))

The formazan product of the MTT tetrazolium accumulates as an insoluble precipitate inside cells as well as being left near the cell surface and in the culture medium. The formazan must be solubilized before recording absorbance readings. A variety of methods have been used to solubilize the formazan product, stabilize the color, avoid evaporation, and reduce interference by phenol red and other culture medium components. Acidification of the solubilizing solution has the benefit of changing the color of phenol red to yellow color that may have less interference with absorbance readings. The pH of the solubilization solution can be adjusted to provide maximum absorbance if sensitivity is an issue; however, other assay technologies offer much greater sensitivity than MTT.

The amount of signal generated is dependent on several parameters including: the concentration of MTT, the length of the incubation period, the number of viable cells and their metabolic activity (Sittampalam, G *et al*., 2004). All of these parameters should be considered when optimizing the assay conditions to generate a sufficient amount of product that can be detected above background. The conversion of MTT to formazan by cells in culture is time dependent.

Protocol for MTT Assay

1. Prepare Ccells and test compounds were prepared in 96-well plates containing a final volume of 100 µl/well.
2. Incubattione for desired period of exposure.
3. Add 10 µl MTT Solution was added per well to achieve a final concentration of 0.45 mg/ml.
4. Incubation fore 1 to 4 hours at 37°C.
5. Add 100 µl of Ssolubilization solution was added to each well to dissolve formazan crystals.
6. Mixing was done to ensure complete solubilization.
7. ARecord absorbance was recorded at 570 nm.
8. Statistical Analysis
9. Absorbance after each dosage concentration was measured in triplicates for both assays and each assay was performed three times. Each dosage concentration

was measured in triplicates for both assays and each assay was performed three times.

RESULTS

Morphological Examination is shown in Figure 2a, 2b, 2c and 2d.

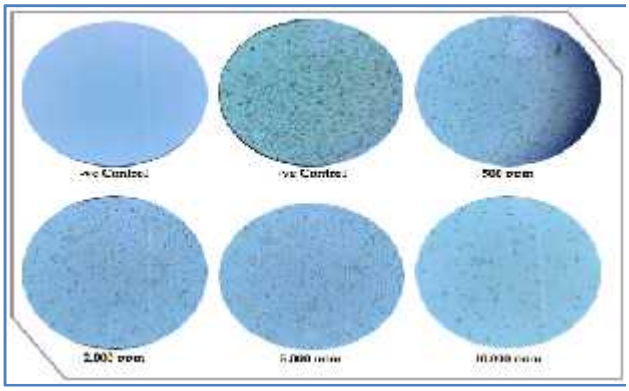

Figure 2a: Day 1

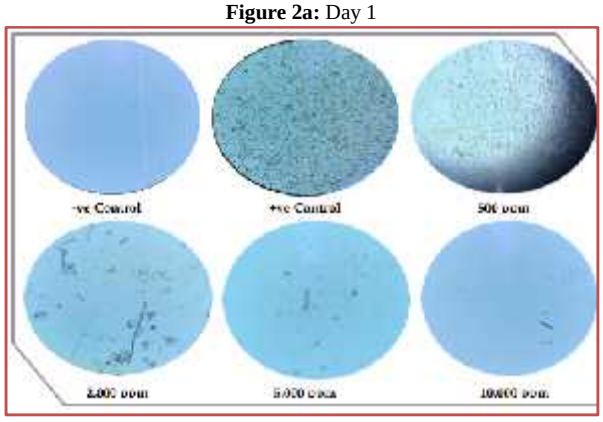

Figure 2b: Day 7

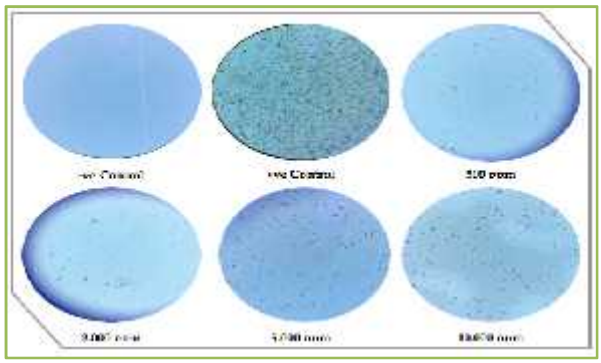

Figure 2c: Day 14

Figure 2d: Day 21

Statistical Analysis

Each dosage concentration was measured in triplicates for both assays and each assay was performed three times.

LDH Assay Results

After initial exposure of 1,4 Dioxane, all cells exhibited cell death upto an average of 12% even for the lowest concentration of 500ppm (Figure 31.A) After 7 days of exposure of the drug, elevated levels of Lactase Dehydrogenase were released in plates at Day 14 and Day 21 as shown in Figure 31.B and 31.C. LDH leakage for concentration 10,000ppm was the highest as expected as shown in Figure 3.D.

LDH leakage for the two low concentrations i.e. 500ppm, 2000ppm showed 12% toxicity. Longer exposure and higher concentration such as 10,000ppm of 1,4 Dioxane showed highest toxicity. Therefore, toxicity was shown to be directly proportional to the concentration as illustrated in Figure 4.

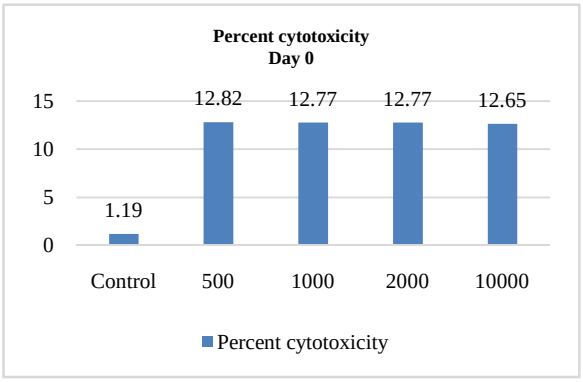

Figure 3 Graph 1 A – Percent cytotoxicity at day 0 using LDH Assay

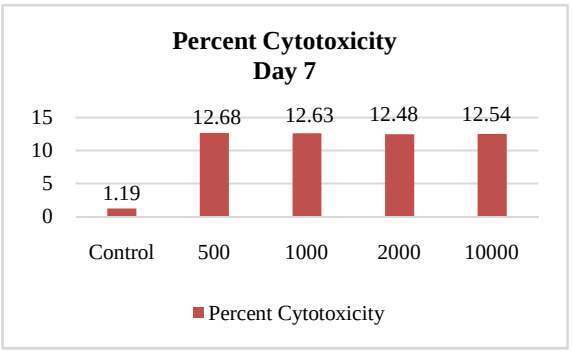

Figure 3 Graph 1 B Percent cytotoxicity at day 7 using LDH Assay

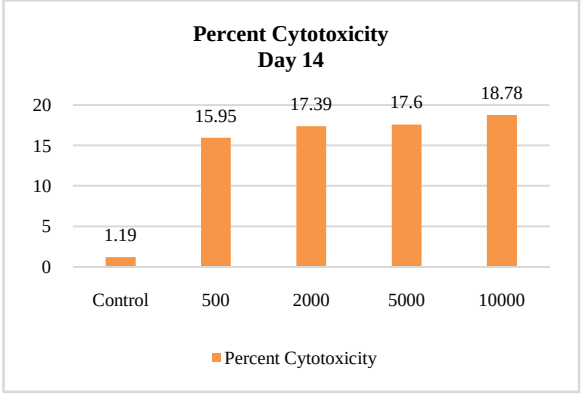

Figure 3Graph 1.C Percent cytotoxicity at day 14 using LDH Assay

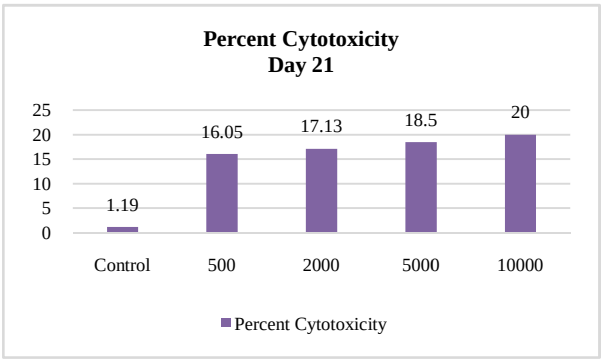

Figure 3Graph 1.D Percent cytotoxicity at day 21using LDH Assay

After initial exposure of 1,4 Dioxane, all cells exhibited cell death upto an average of 12% even for the lowest concentration of 500ppm (Figure 1.A) After 7 days of exposure of the drug, elevated levels of Lactase Dehydrogenase were released in plates at Day 14 and Day 21 as shown in Figure 1.B and 1.C. LDH leakage for concentration 10,000ppm was the highest as expected.

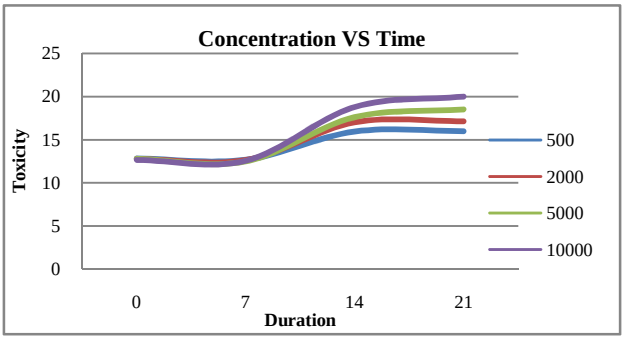

Figure 4 Graph 2 - Represents toxicity as measured by LDH Assay versus duration

From the above graph we can conclude that LDH leakage for two concentrations i.e. 500ppm, 2000ppm showed 12% toxicity. Longer exposure and high concentration such as 10,000ppm of 1, 4 Dioxane showed highest toxicity. Therefore, concentration is directly proportional to toxicity.

MTT Assay Results

The outcome of this assay indicated that the cell viability was higher at Day 21 compared to the other three durations which were Day 1, Day 7 and Day 14 (as shown in Figure 5) which means that the cells were more viable after exposure to the highest concentration of 1,4 Dioxane; which was non-logic. This assay was repeated thrice, and the outcome was same for all. Theoretically, this assay's result was not as acceptable as LDH assays.

According to figure 3, the outcome of this assay indicates that the cell viability is more at Day 21 compared to the other three days which are Day 1, Day 7 and Day 14 which means that the cells are more viable after exposure to the highest concentration of 1,4 Dioxane; which is illogical. The assay was repeated thrice, and the outcome was same for all. Theoretically, this assay's result isn't as acceptable as LDH assays were.

DISCUSSION

In previous literature, toxicity was not observed in lowest concentration such as 500ppm. However, we observed minimum cell death of 12% immediately after exposure and maximum cell death of 16% after 21 days of exposure. This percent toxicity was higher than expected for the lowest concentration. Therefore, further trails and research should be done for concentrations equal and lower to 500ppm to investigate the toxicity it can possibly cause.

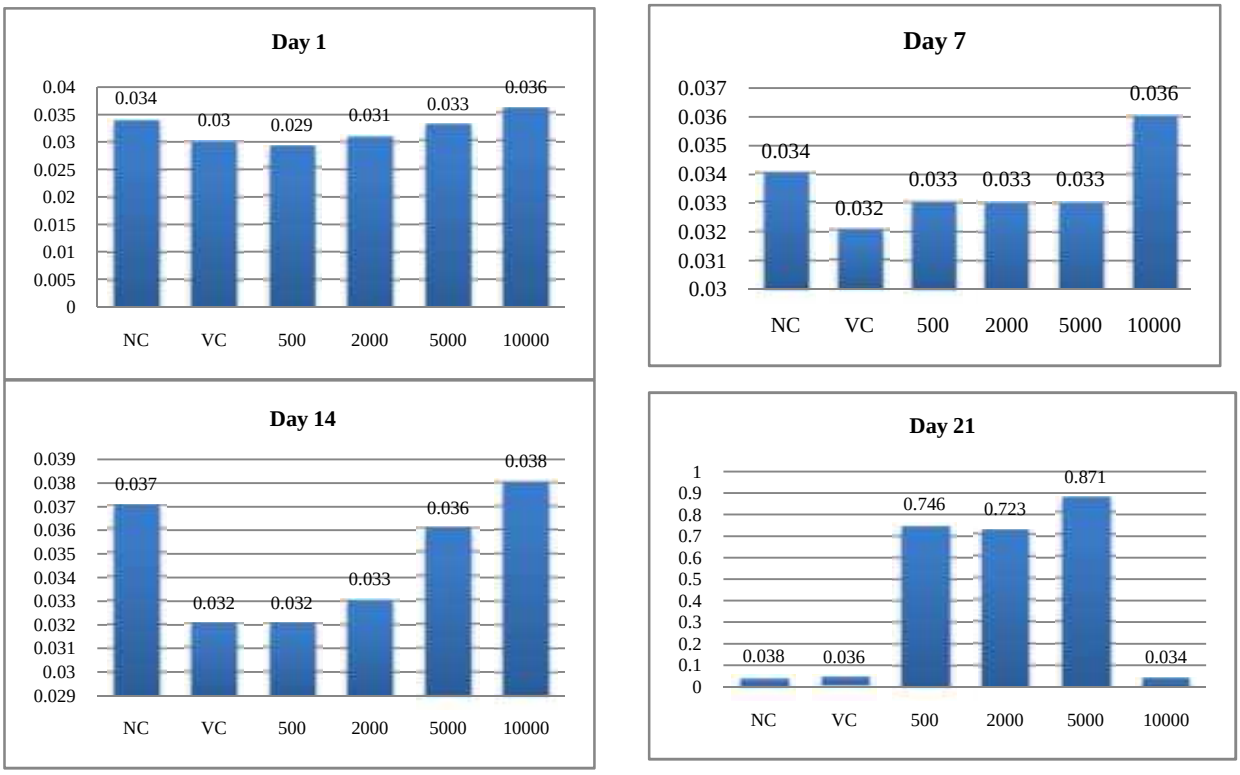

Figure 5Graph 3 Outcome of MTT assays after exposure of HEP10 cell line to different concentrations of 1, 4 Dioxane atfor different durations

Furthermore, tTheoretically, the signal in the LDH assay is inverse (higher signal means lower viability) to other assays like MTT (where higher signal means higher viability). According to our results, the highest toxicity was observed in the highest concentration (10,000ppm) using LDH assay as expected whereas for MTT assay, highest viability was also observed in the highest concentration.

Previous studies also show that MTT assay is rarely the best option for estimating the number of viable cells in vitro. Chemical interference and lack of sensitivity is discovered to be the limitation of MTT Assay according to few studies. As it is known that the MTT assay is easy and inexpensive enough that it could be used as a preliminary method for screening. It should not be used as primary evidence for cytotoxicity or anti-proliferative activity because it measures mitochondria as an indirect measure of cell count.

CONCLUSION

We ran both cell viability using MTT and LDH analysis, but we found the same compound shows higher levels of LDH release as well as to the cell viability.

1. The current study had further supported the previous claims against the use of 1,4 Dioxane and confirmed its cytotoxic effect. Additionally,Llower concentration of 1,4 dioxane has showed relatively high cell toxicity which was not expected or witnessed in previous literature studies. We would recommend The study of the possiblethe toxic effect of much lower concentrations of 1,4 dioxane on the cells is neededneed to be further investigated. Furthermore, this study has supported the previous suggestions regarding the efficiency of the use of MTT Assay for checking the cell viability being not entirely reliable due to non-specific intracellular reduction of tetrazolium that leads to underestimation of the cytotoxicity results.
2. MTT assay is commonly used to assess the cellular cytotoxicity. However, there have been some reports insisting that MTT assay exhibited non-specific intracellular reduction of tetrazolium which led to underestimated results of cytotoxicity. Therefore, MTT Assay cannot entirely be relied on for checking cell viability.

References

Atsdr.cdc.gov, (2012). ATSDR - Public Health Statement: 1,4-Dioxane. [online] Available at: http://www.atsdr.cdc.gov/PHS/PHS.asp?id=953&tid=199 [Accessed 22 Jan. 2016].

Atsdr.cdc.gov, (2012). ATSDR - Toxicological Profile: 1,4-Dioxane. [online] Available at: http://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=955&tid=199 [Accessed 20 Jan. 2016].

Cali, J., Ma, D., Sobol, M., Simpson, D., Frackman, S., Good, T., Daily, W. and Liu, D. (2006). Luminogenic cytochrome P450 assays. Expert Opinion on Drug Metabolism & Toxicology, 2(4), pp.629-645.

EPA Technical Factsheet (2014), http://www2.epa.gov/ sites/ production/files/2014- 03/documents/ffrro_factsheet_ contaminant_14-dioxane_january2014_final.pdf [Accessed 20 Jan. 2016]

EPA, (2000). 1,4-Dioxane (1,4-Diethyleneoxide) | Technology Transfer Network Air Toxics Web site | US EPA. [online] Available at: http://www3.epa.gov/airtoxics/hlthef/dioxane.html [Accessed 22 Jan. 2016].

Kosswig, K. (2000). Surfactants. Ullmann's Encyclopedia of Industrial Chemistry.

Mohr, T., Stickney, J. and Diguiseppi, B. (2010). Environmental investigation and remediation. Boca Raton [FL]: CRC Press.

Nannelli A, De Rubertis A, Longo V, Gervasi PG. (2005). Effects of dioxane on cytochrome p450 enzymes in liver, kidney, lung and nasal mucosa of rat. Arch Toxicol 79:74-82.

Ngo, T., Brillhart, K., Davis, R., Wong, R., Bovaird, J., Digangi, J., Ristow, J., Marsh, J., Phan, A. and Lenhoff, H. (1987). Spectrophotometric assay for ornithine decarboxylase. Analytical Biochemistry, 160(2), pp.290-293.

Promega, (2015). P450-Glo™ Assays Protocol. [online] Available at: https://worldwide.promega.com/ resources/ protocols/technical-bulletins/101/p450-glo-assays-protocol/ [Accessed 22 Jan. 2016].

Safe Cosmetics, (2008). 1,4-dioxane - Safe Cosmetics. [online] Available at: http://www.safecosmetics.org/get-the-facts/chemicals-of-concern/14-dioxane/ [Accessed 20 Jan. 2016].

Sigel, A., Sigel, H. and Sigel, R. (2007). The ubiquitous roles of cytochrome P450 proteins. Chichester: John Wiley. Available at: http://www.ncbi.nlm.nih.gov/books/NBK53196/

Sigmaaldrich.com, (2016). MSDS - 296309. [online] Available at: http://www.sigmaaldrich.com/ MSDS/ MSDS/DisplayMSDSPage.do?country=AE&language=en&productNumber=296309&brand=SIAL&PageToGoToURL=http%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Fsial%2F296309%3Flang%3Den [Accessed 20 Jan. 2016].

Sittampalam GS, Coussens NP, Nelson H, Arkin M, Auld D, Austin C, Bejcek B, Glicksman M, Inglese J, Iversen PW, Li Z, McGee J, McManus O, Minor L,Napper A, Peltier JM, Riss T, Trask OJ Jr., Weidner J, editors. (2004). Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences.

Toumi, H. (2010). Qatari shampoo ban to continue as talks fail. Gulf News Qatar. [online] Available at: http://gulfnews.com/news/gulf/qatar/qatari-shampoo-ban-to-continue-as-talks-fail-1.658336 [Accessed 20 Jan. 2016].

1,4-Dioxane [MAK Value Documentation, 2003]. (2012). Annual Thresholds and Classifications for the Workplace.

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