Phenol as a positive control in cytotoxicity test methods

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Abstract

Cytotoxicity test is a primary indictor of biocompatibility and safety of medical devices. The ISO 10993 has recommended several in vitro cytototoxicity test methods and parameters. One of them is possibility to apply phenol as a positive control. Phenol is cytotoxic to cell and tissue cultures over determined concentrations. The paper describes errors generated in experimental results if phenol is used for long period in cytototoxic test methods. The ARPE-19 and L929 cell cultures are prepared in 96 wells plate following ISO 10993 recommendations and exposed to positive, negative and test samples. Different concentrations of phenol (0.025, 0.1, 0.2. 0.4, 1.6 mg/ml as well as 1x (1.6 mg/ml), 3x, 66.6% of 1x and 3x) are prepared with culture medium and used in different experimental plans. The cell cultures were exposed, following direct contact cytotoxicity method, directly to phenol concentration for 30 and 60 minutes and then grown for 24 and 72 hours, or were, following extract contact cytotoxicity method, incubated 72 hours with phenol concentrations. The cell culture viability was measured using the MTT and XTT assay. The data are analyzed statistically and errors generated due to phenol samples were detected. The results showed the cell culture had lost their viability (<1%) at 1.6 mg / ml concentration of phenol on 30 minutes exposure. On short period exposure (up to 60 minutes in this study), there were no effects on viability of cell cultures of neighboring wells of a culture plate. However, where cell cultures were incubated for a long period, 72 hours, with phenol, the cell culture of neighboring wells had also lost their viability. This lost decreases as far as wells are from the wells that contained phenol. The results showed the L9292 cell cultures were more sensitive to phenol than ARPE-19 cell cultures. The study concludes that including phenol presence as a positive control for long period in an experimental plan can generate errors in data; which concludes into false positive for a test sample.

Keywords

Phenol, Cytotoxicity

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Introduction

Cytotoxicity test is a primary indicator of biocompatibility and safety of medical devices; medical contrivance, implant, in vitro reagent, or other similar or related device, including a component part, or accessory etc (Li et al., 2015; Williams, 2008) . The medical devices are applied routinely in the diagnosis of disease, cure, mitigation, treatment, or prevention of disease or other health conditions (Werginz and Rattay, 2015). Most of them are in direct contact with human body. The ISO 10993 guidelines describe various test methods to assess in vitro cytotoxicity of medical devices ((10993-12:2007 Parte 12 preparation of samples and reference materials, UNE-EN ISO 10993-5:2009 Part 5 in vitro cytotoxicity test, Part 5 section C.2.3.3 Verification of the quality of analysis (II); blank). For these methods, the cell culture quality is determined, and then they are exposed to test sample or extract of test sample either directly or through indirectly for a period of time. Further cell culture is grown for a period and at the end, cell culture viability or mortality is measured to evaluate cytotoxicity potential of test sample. Thus, these methods are designed to determine the biological response of cells, grown in vitro conditions, using appropriate experimental parameters. Each test method consists of three groups; a test samples group, a positive control group and a negative control group. The control groups evaluate a cell culture quality by detecting cell culture uniformity and its response to differentiate between non cytotoxic (negative control) and cytotoxic (positive control) test samples. These evaluations are crucial steps of protocol of any method. The ISO guidelines have recommended various substances which can be used as a positive control. The liquefied phenol which meets USP testing specifications is one of them.

Phenol has molecular formula C6H6O, weight 94.11 g/mol and storage temperature 2 - 8 °C (National Center for Biotechnology Information. PubChem Substance Database; SID=24899028, https://pubchem.ncbi.nlm.nih.gov/substance/24899028.). The liquified phenol is a solution of phenol containing ~10% water. This is a high quality pharmacopoeia product that meets the testing specifications of USP (United States Pharmacopoeia). The phenol is volatile organic substance and it is used in several cytotoxicity studies (Barron, 2002; Quan et al., 2008). Several reports have demonstrated that it produces toxic effect, including cytotoxicity to cell cultures (Quan et al., 2008). The optimum concentration of it which produces cytotoxicity depends on the cell types and intensity depends on experimental parameters (Alghamdi et al., 2014; Manju et al., 2011; Watson and Zibadi, 2013; Yager et al., 1990). However, in our knowledge, it was not reported that in experimental parameters, it can be cytotoxic for cell cultures of neighboring wells of a culture plate. After an extensive search there are not references on this topic (used search key words: phenol, positive control, experimental parameters, cell culture, neighboring wells, culture plate, ISO norms, phenol evaporation, cytotoxicity, and bibliography sources: PubMed, Google search, Google scholar, Scopus, Web of Science, Sciencedirect National Service Center for Environmental Publications (NSCEP) and many others)

The purpose of this study is to describe possible errors generated in experimental data if phenol (as a positive control) is used under certain parameters in cytotoxic test methods.

Materials and methods

Cell culture

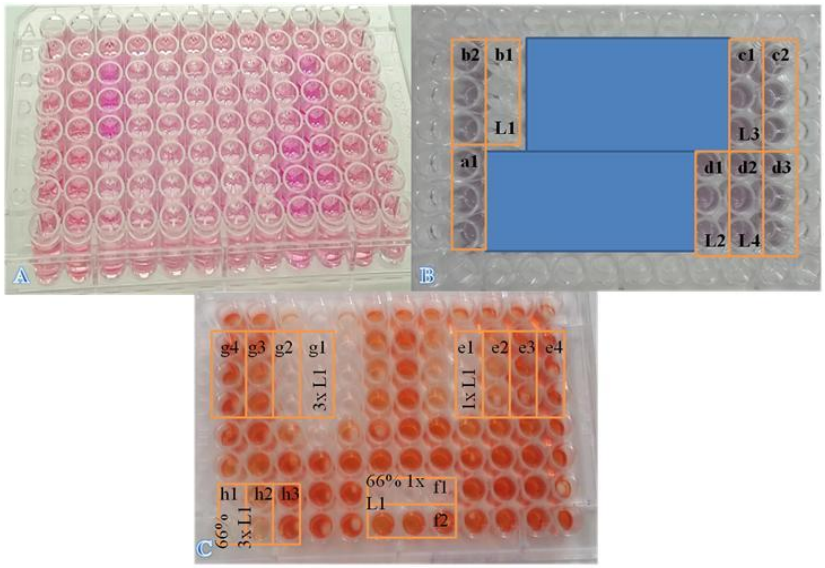


Figure 1. Effect of phenol on cell cultures and cell cultures of neighboring wells of a 96 well culture plate. Sketch of the experimental plans (A, B and C). A) The ARPE-19 cell cultures were exposed to 1.6, 0.4, 0.2 and 0.025 mg/ml (L1, L2, L3 and L4) concentrations of phenol, and cell culture medium (group a1, b2, c2 and d3) for 30 or 60 minutes. B) The MTT assay to measure cell culture viability for each well of the culture plate at the end of each growth period. The area covered by dark blue is for test samples which are not discussed in this article. C) Cell cultures were exposed to 1x and 3x of phenol concentration (1.6 mg/ml = 1x (L1)) for 72 hours. The XTT assay was performed to measure cell culture viability for wells contained phenol and their neighboring wells (groups e1, e2, e3 and e4 for determining effect of L1, groups f1 and f2 for 66% of L1, groups g1, g2, g3 and g4 for 3x of L1 and groups h1, h2 and h3 for 66% of 3x of L1).

Cell culture plate preparation

The 96 well cell culture plates were used for performing experiments of this study. Single cell suspensions of the L929 and ARPE-19 cells were prepared and cell viability were counted using Trypan blue exclusion method by TC20TM automatic cell counter (Bio-Rad Laboratories). The cell suspensions showing ~ 90% viability, were selected for cell seeding in cell culture plates. In one set the ARPE-19 cells were seeded using multichannel pipette in cell density of 10 x 10^3 cells per well in 200 µl of cell culture medium and grown for 7 days in standard culture conditions. The cell culture medium was renewed at every 2-3 days intervals. At 7 days the cell culture was synchronized for cell cycle by incubating 10% FBS deprived cell culture medium for 24 hours. In another set, the L929 and ARPE-19 cells were seeded using multichannel pipette in cell density of 5 x 10^3 cells per well in 50 µl of corresponding cell culture mediums and grown for 24 hours in standard culture conditions. Two different cell seeding densities; 10 x 10^3 cells per well and 5 x 10^3 cells per well, were selected for the experimental purpose to verify the findings of this study.

Exposure of phenol to cell cultures

The ARPE-19 and L929 cell cultures were applied for this study. For this purpose the ARPE-19 cell line (human retinal pigment epithelial cells) and L929 cell line (mouse fibroblast) were obtained from American Tissue Culture Collection (ATCC) and grown in the DMEM / F12 and DMEM culture media respectively. The culture media were supplemented with 10% fetal bovine serum (FBS) and 1% of antibiotic and antimycotic (100 U / ml Penicillin, 100 µg / ml Streptomycin and 0.25 µg / ml of Amphotericin B). These cell cultures were maintained in 75 cm2 flasks in standard culture conditions of 37 ºC and 5% CO2. The cell culture mediums were replaced with fresh culture medium at every two day. When the cell culture growth reached to 80-90 % confluence in flask, they were trypsinized applying 0.05% Tripsin-EDTA solution and sub-cultivated.

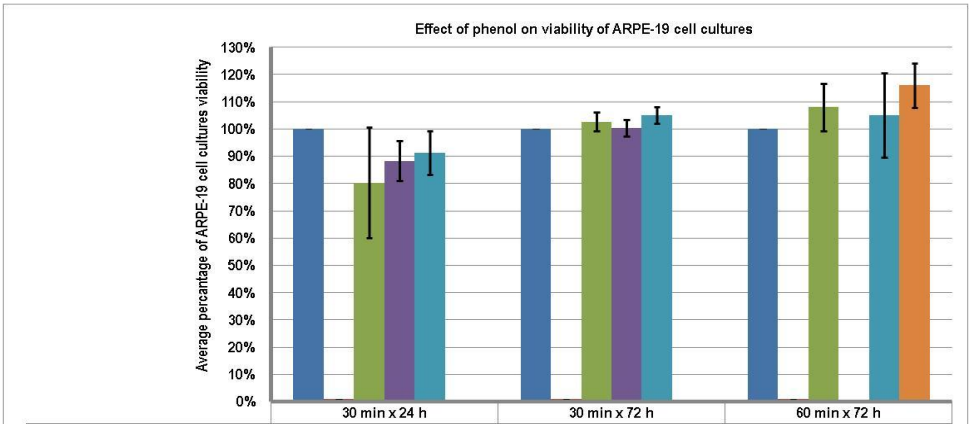
The ARPE -19 cell cultures grown for 7 days were washed 3 times using corresponding FBS deprived cell culture medium. Each time for washing, the 200 µl of cell culture medium was added in each well using multichannel pipette and the culture plate was inverted up to down slightly vigorously to discard whole medium from each well. After washing the cell cultures were incubated with 200 µl of different concentrations (L1, L2, L3 and L4) of phenol. The liquefied phenol, lot # MKBX0568V, which meets USP testing specifications was bought from Sigma-Aldrich (Saint Louis, MO, USA) and prepared in a stock solution of 1.6 mg / ml (L1) in cell culture medium and further diluted for preparing concentrations of 0.4 mg / ml (L2), 0.2 mg / ml (L3), and 0.025 mg / ml (L4). In another experiment L4 was replaced with 0.1 mg / ml concentration of phenol. The cell cultures were exposed with phenol for 30 and 60 minutes. Then they were washed and grown for 24 and 72 hours. At the end of cell culture growth, the MTT assay was performed to measure the cell culture viability.

For another set of experiments, the L929 and ARPE-19 cell cultures grown for 24 hours were washed using corresponding mediums as described previously. Then they were incubated with 1x and 3 x concentrations of phenol (1.6 mg / ml) and 66.6 % of 1x and 3x concentrations in standard culture conditions for 72 hours. In this case before the incubations, the phenol concentrations were stirred for 24 hours at 37 ºC in inert bottles accomplishing the recommendations of ISO guidelines. At the end of cell culture growth, the XTT assay was performed to measure the cell culture viability.

In both cases, rest of wells of each plate was incubated with fresh cell culture medium or cell culture medium stirred for 24 hours at 37 ºC or negative controls.

MTT and XTT assay for cytotoxicity measurement

At the end of growth period, the cell culture medium from each well was removed, washed 1-2 times and cell culture viability was measured applying the MTT or XTT assay. For the MTT assay, the 10% fresh MTT solution from a stock solution of 5 mg / ml of the MTT in cell culture medium without phenol red was prepared and 50 µl of this MTT solution was added in each well. The cell culture plate was transferred to incubator set at standard culture conditions for 2 hours. Following 2 hours, the MTT solution was removed and the formed crystals with cells of cell culture of each well were solubilized in 100 µl of an acidic solution of the isopropanol (0.1 N HCL in 2-propanol, Sigma; lot # BCBQ9033V). The repetitive pipetting using multichannel pipette was performed for solubilization and the formed solution of each well was transferred to a fresh flat bottom 96 well cell culture plate. The absorbance of



	30 min x 24 h	30 min x 72 h	60 min x 72 h
Fresh DMEM / F12 culture medium	100%	100%	100%
1.6 mg/ml	1%	1%	1%
0.4 mg/ ml	80%	103%	108%
0.2 mg/ml	88%	100%	0
0.1 mg/ml	91%	105%	105%
0.025 mg/ml	0	0	116%

Figure 2. Average percentages of the ARPE-19 cell culture viability on 30 and 60 minutes exposures of different concentrations of phenol, 1.6, 0.4, 0.2 and 0.025 mg/ml (L1, L2, L3 and L4), and posterior incubated for 24 and 72 hours for cell growth in standard culture conditions (30 min x 24 h, 30 min x 72 h and 60 min x 72 h). The MTT assay was performed to measure the cell culture viability at the end of each cell culture growth period. Data were calculated for obtaining percentages of cell culture viability and statistically analyzed. Percentage of cell culture viability for fresh DMEM / F12 culture medium was set at 100% to compare the results. The value zero (0) represents those groups which were not tested. .

solution of each well was measured at a wavelength of 570 nm using a spectrophotometer SpectraMax® M5. The absorbance at 650 nm was the background absorbance, and was subtracted from the readings at 570 nm to obtain the final optical density (OD).

The XTT assay was performed following the protocol of manufacturer (Roche; Cell Proliferation Kit II (XTT) Lot # 11891000). A fresh mixture of XTT labeling reagent (5 ml) and the electron coupling reagent (0.1 ml) was prepared and the 50 µl of this mixture was deposited in each well. The cell culture plates were transferred to incubator for 2 hours and the absorbance reading of each well was measured using the spectrophotometer SpectraMax® M5 at a wavelength of 490 nm. The absorbance at 630 nm was the background absorbance, and was subtracted from the readings at 490 nm to obtain the final optical density (OD).

Data analysis

Data was analyzed following ISO guidelines 10993-5. Background absorbance OD was subtracted for obtaining final value of OD. The formula applied was for measuring the percentage viability was 100 x OD (test sample) / OD (reference sample). The percentage presented in the graph is mean of percentages obtained in each case. Data showed statistically significant difference between one group to another group if p=0.05. Each group is in triplicate (n=3).

Results

The figure 1 showed the experimental plans applied for measuring the concentrations of phenol lethal to ARPE-19 cell cultures and to demonstrate their effects on cell cultures of neighboring wells. Similar experimental plans were executed with L929 cell cultures.

There were four different groups in the study for comparison:

- Groups for 1.6, 0.4, 0.1 and 0.025 mg / ml of phenol.
- Groups for 1x and 3x concentrated solutions of 1.6 mg/ml of phenol.
- Groups for 66% of 1x and 3x concentrated solutions of 1.6 mg/ml of phenol.
- Rest all the groups contained only culture medium to measure effects of phenol evaporation on cell cultures of neighboring wells.

Phenol concentrations lethal to ARPE-19 cell culture

The observations during exposure period showed that the wells containing phenol had acquired bright red colour and visible distinct from other wells (figure 1 A). Subsequent the MTT assay plate (figure 1 B) showed those wells were containing reduced quantity of formazan crystals (dead cells). Further, the data analysis (figure 2) showed that the ARPE-19 cell culture viability was <1%, 80% 88% and 91% for concentrations 1 (group L1), 0.4 (group L2), 0.2 (group L3) and 0.1 (group L4) mg / ml of phenol for 30 minutes exposure and posterior 24 hours cell culture growth (30 min x 24 h). For same concentrations and exposure period, the cell culture viability was <1%, 103%, 100% and 105% for posterior 72 hour cell culture growth (30 min x 72 h). Further the results showed that for 60 minutes exposure and posterior 72 hours cell growth (60 min x 72 h), the cell culture viability was 1%, 108%, 105% and 116% for previous serial of concentrations (L1, L2, L3 and L4) of phenol. .

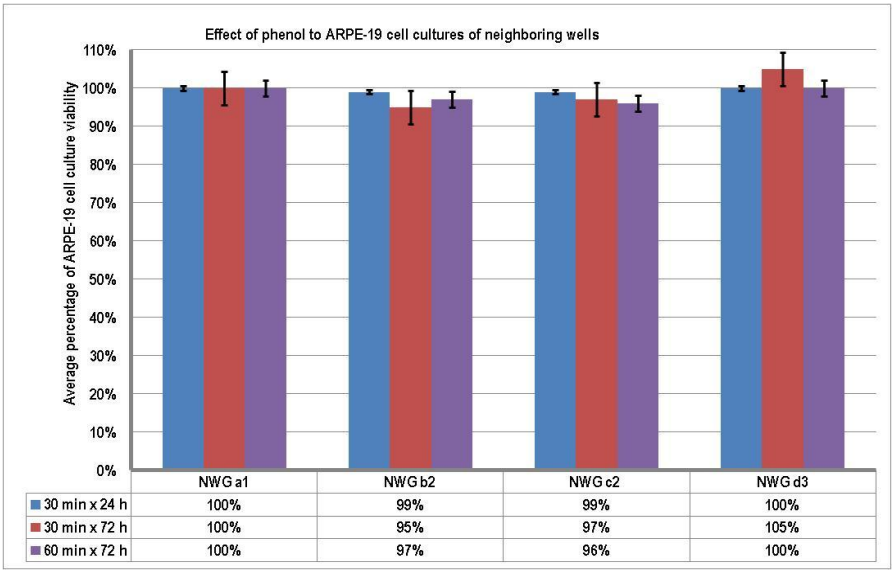
Vortex mixing for phenol concentration preparation

Two sets of the lethal concentration (1.6 mg / ml) of phenol were prepared; one was mixed each time before adding in a culture plate (set 1) and another one was mixed only at starting of experiment (set 2). The results showed that in first set the APRE-19 cell culture viability was reduced to <1%, however in second set, the viability was 33% which sometime could reached to >70% (data not shown) (60 min x 30 h).

Effect on neighbouring well cell cultures

Short period phenol incubation effect

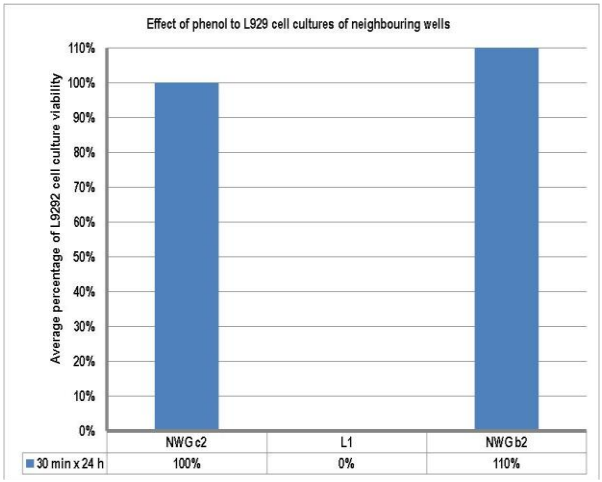
The ARPE-19 cell cultures were exposed to phenol concentrations (L1, L2, L3 and L4) for 30 and 60 minutes, posterior they were grown in culture medium at standard cultures conditions. The cell culture viability was measured at the end of growth periods of 24 and 72 hours. The results (figure 3) showed that on 30 minutes exposure the cell cultures viability of neighboring wells, groups a1, b1, c2 and d3, were, respectively, 100%, 99%, 99% and 100 % for 24 hours of growth (30 min x 24 h), and 100%, 95%, 97% and 105% for 72 hours of growth (30 min x 72 h). For exposure of 60 minutes followed by posterior 72 hours of growth (60 min x 72 h), it was 100%, 97%, 96% and 100% respectively. The data was calculated setting cell culture viability of the group only with culture medium at 100% (reference group).



	NWG a1	NWG b2	NWG c2	NWG d3
30 min x 24 h	100%	99%	99%	100%
30 min x 72 h	100%	95%	97%	105%
60 min x 72 h	100%	97%	96%	100%

Figure 3. Effect of phenol on viability of the ARPE-19 cell cultures of neighboring wells of a 96 well plate (groups a1, b2, c2 and d3). The ARPE-19 cell cultures of wells b1, d1, c1 and d2 were exposed to 1.6, 0.4, 0.2 and 0.025 mg/ml (L1, L2, L3 and L4) concentrations of phenol for 30 or 60 minutes. Posterior the cell cultures were grown for 24 and 72 hours in culture medium at standard culture conditions. The MTT assay was performed to measure the cell culture viability of each group (a, b, c and d) at the end of each growth period. Data was calculated for obtaining average percentage of cell culture viability for each group and statistically analyzed for differences. The cell culture viability of group a was set at 100% to compare the results. NWG refers to Neighboring Well Groups.

Thus, these results demonstrated that cell cultures viability of neighboring wells was ~ 100%. For further confirming the results, different sets of experiments were performed; in one the reference group was set in another plate which did not contain any wells with phenol. In another set of experiment, the phenol groups L2, L3 and L4 was removed from culture plate to find changes in cell culture viability of right and left groups of culture plates, and in third set the 2x and 3x concentrations of phenol were tested. The cell culture viability was measured and compared between group a, b, c and d. The data of above experimental conditions was not shown because it was found there were no changes in trends of the results.



	NWG c2	L1	NWG b2
30 min x 24 h	100%	0%	110%

Figure 4. Effect of phenol on L929 cell cultures of neighboring wells of a 96 well plate. The L929 cell cultures were exposed to 1.6 mg /ml (L1 or b1) concentrations of phenol for 30 minutes and then cell cultures were grown for 24 hours in culture medium at standard culture conditions. At the end of the growth period, cell culture viability of each group (a, b, c and d) was measured by the MTT assay. Data was calculated for obtaining average percentage of cell culture viability for each group and statistically analyzed for differences. The cell culture viability of group c2 was set at 100% to compare with cell culture viability of rest of wells. NWG refers to Neighboring Well Groups.

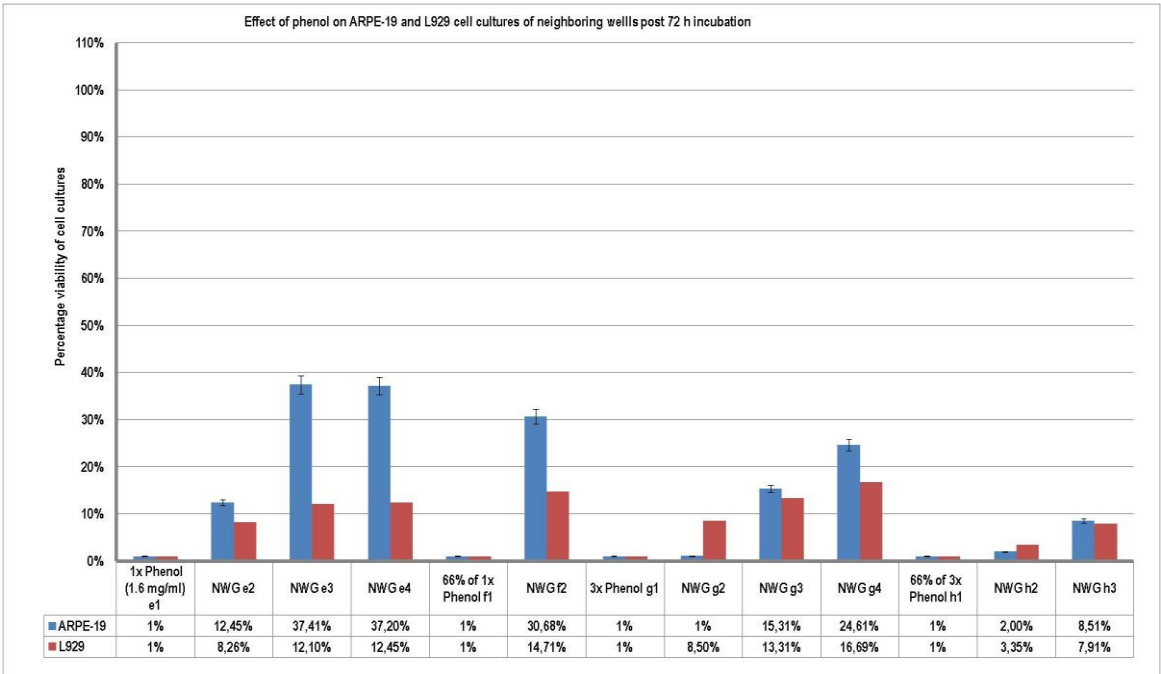
An experiment was performed with L929 cell cultures grown in cell culture plate including only groups b2, L1 and c2. The results (figure 4) showed L929 cell culture viability was 100% (group c2), 0% (group L1) and 110% (group b2) respectively after 30 minutes exposure with phenol concentration L1 and posterior growth of 24 hours. For comparing the data, in this case the group c2 (culture medium) was set as a reference (100%).

Long period phenol incubation effect

The figure 1 B and C showed the experimental plan which was performed and the results which were obtained at the end of the execution of experimental plan. The observation showed that the groups with phenol had acquired very mild visible red color and were not easy to separate from other groups. But as time passed, it was possible to separate them because of presence of dead cells and less consumption of culture medium. Posterior the XTT assay plate (figure 1 C) clearly showed that those wells including the neighboring wells which were affected due to presence of phenol in culture plate (dead cells). Further, analysis of data obtained at 72 hours of the culture growth posterior to exposure of 1 x concentration of phenol (L1), the results showed (figure 5) that the cell culture viability was <1%, 12.45%, 37.41% and 37.20% (for ARPE-19), <1%, 8.26%, 12.10% and 12.45% (for L9292) for group e1, e2, e3 and e4. On exposure of 66% of 1x phenol, the culture viability was <1%, 30.68% (for ARPE-19) and <1% and 14.71% (for L929) for group f1 and f2 respectively. On exposing the cultures to 3x concentration of phenol (L1), the culture viability was <1%, <1%, 15.31 and 24.61% (for ARPE-19), and <1%, 8.50%, 13.31% and 16.69% (for L929) for group g1, g2, g3 and g4. When cultures were exposed for 66% of 3x concentration of phenol (L1), the cell culture viability was <1%, 2% and 8.51% (for L9292) and <1%, 3.35% and 7.91% (for L9292) for groups h1, h2 and h3 respectively.

Discussion and Conclusion

A medical device is safe for patient, to certify it is a huge concern for R&D personnel, manufacturers and sellers as well as for health authorities (Bollen and Svendsen, 1997) . Cytotoxicity test method drives the fate of a medical device for its safe application. There are several cytotoxicity test methods, and many of them are included in ISO guidelines. The guidelines also have recommendations for parameters applied for experimental plans including cell cultures quality, negative and positive controls. It is for bringing some sort of uniformity in experimental procedures and parameters executed by different certification authorities (European, British, Indian, Australian, Japanese etc territory based standards) and concluding results to ensure that materials, products, processes and services are fit for their purpose



	1x Phenol (1.6 mg/ml) e1	NWG e2	NWG e3	NWG e4	66% of 1x Phenol f1	NWG f2	3x Phenol g1	NWG g2	NWG g3	NWG g4	66% of 3x Phenol h1	NWG h2	NWG h3
ARPE-19	1%	12,45%	37,41%	37,20%	1%	30,68%	1%	1%	15,31%	24,61%	1%	2,00%	8,51%
L929	1%	8,26%	12,10%	12,45%	1%	14,71%	1%	8,50%	13,31%	16,69%	1%	3,35%	7,91%

Figure 5. Effect of phenol on cell cultures of neighboring wells of a 96 well plate. The ARPE-19 and L929 cell cultures were exposed to 1x and 3x of phenol concentration (1.6 mg / ml =1x (L1)) for 72 hours and then XTT assay was performed to measure cell culture viability of wells contained phenol and their neighboring wells; group e1, e2, 23 and e4 for determining effect of L1, group f1 and f2 for 66% of L1, group g1, g2, g3 and g4 for 3x of L1 and group h1, h2 and h3 for 66% of 3x of L1. Data was calculated for obtaining average percentage of cell culture viability for each group and statistically analyzed for differences. The cell culture viability of another 96 cell culture plate which does not contain phenol was set at 100% to compare the results. NWG refers to Neighboring Well Groups.

(Mellström, 1991; Parsons, 1999). Among several substances recommended as positive controls, the guidelines recommend the liquified phenol accomplishing the testing specifications of USP (International standard ISO 10993-5, Biological evaluation of medical devices. 1992. Part 5: Tests for cytotoxicity: in vitro methods.). However, the results of this study showed that phenol is not a good positive control for an experimental plan executed with certain parameters (as defined in this experimental work) and it confirmed that it can produce errors in experimental data.

The ARPE-19 and L929 cell cultures were selected for this study because ARPE-19 cells are human epithelial cells and L929 cells are mouse fibroblasts cells. These cell lines are currently in use as part of our routine research works for analyzing different tests (pharmaceutical products and medical devices such as perfluorocarbon liquid, neuroprotective agents, stem cell based neuroprotection, tissue engineering). The comparison of results obtained using these two cell types will support to solve the doubts about sensitivity of cells having different origins, human and mouse, against a toxic product. The L929 cell cultures are popular for testing medical devices for cytotoxicity and most of certification authorities use this cell line for certifying safety concern of medical devices (International standard ISO 10993-5, Biological evaluation of medical devices. 1992. Part 5: Tests for cytotoxicity: in vitro methods.). It is irrespective of the location in the human body where the medical device is going to be used and what type of cells or tissue will come in touch with the medical device. Notable cell cultures show different sensitivity and it is related to cell types (Johnson et al., 1983). The ARPE-19 cells represent retinal pigment epithelial cells which could come under influence exerted by medical devices used in eye surgery. The medical devices used during intraocular surgery are not in touch with RPE cells but with optic nerve fibers layer or ganglion cells or inner limiting membrane (Hynes and Lavik, 2010). Both cultures are prepared applying ISO recommendation and the figure 1 show the experimental plan and results.

For obtaining an optimum concentration of phenol which is toxic for cell cultures, a dose dependent cytotoxicity study was performed. The results showed that ARPE-19 cell cultures lost their viability (<1%) in 24 hours at a concentration of 1.6 mg/ml which were exposed for 30 minutes. The evaluation of cytotoxic effect at 24 hours was selected due to recommendation of the ISO guidelines. The 30 minutes exposure was selected considering phenol potential to produce cytotoxicity to ARPE-19 cell cultures and testing perfluoro-n-octane test samples used as a medical device in retinal surgery (preliminary studies). The results confirmed that the ARPE-19 cell cultures retained their viability >70% at concentrations of 04, 0.2, 0.1 and 0.025 mg / ml. According to the ISO norms, it is consider non toxic if a cell culture maintains viability >70%. Thus, the phenol cannot be used in these concentrations as a positive control in an experimental plan if ARPE-19 cell cultures are exposed only for 30 minutes. Further results obtained at 72 hours growth or for 60 minutes exposure followed by 24 and 72 hours growth confirmed this conclusion. Subsequent study showed the impact of proper preparation of a phenol concentration. The results showed that if it is not mixed properly with culture medium before adding in a well each time, the ARPE-19 cell culture could retain its viability up to 33% and sometime it could reach >70% representing a non toxic result (in accordance to ISO 10993 guidelines). Thus, it could produce failure in the experimental plan with test samples.

The phenol has a property to evaporate; therefore, it was necessary to look if it affects the cell cultures of neighboring wells. For this purpose, several studies were performed with ARPE-19 and L929 cell cultures. The ARPE-19 cell cultures were exposed for 30 and 60 minutes for the concentration of 1.6 mg / ml of the phenol, and then grown for 24 and 72 hours. At end of the growth, the viability of cell cultures of neighboring wells was also measured for cytotoxic effect of phenol. The results showed that the cell cultures of all the neighboring wells retained their viability ~100 %, although phenol exposed cell cultures lost their viability. This confirmed that for short period of exposure, 30 and 60 minutes, there were no effects of phenol evaporation to cell culture viability of neighboring wells. Several other experimental plans with the ARPE-19 cell cultures and their results as described in section “result” including using 2x and 3x concentrations of 1.6 mg / ml (1x) of phenol confirmed this conclusion. For further confirmation, the L929 cell cultures were used and the results confirmed that the cell culture of neighboring wells retained their viability at 100% (no effect of phenol exposure for short period).

The results obtained exposing the cell cultures for long period, 72 hours, showed that the L929 cell cultures are more sensitive for cytotoxic effect of phenol and there are effects on viability of cell cultures of neighboring wells. In case of the group e and g, the cell culture viability was measured up to the 4th wells (e4 and g4) which are 3 wells far from those wells that contain phenol (e1 and g1). The results showed that the viability of cell culture of

these wells were 37.20% (e4) and 8.51% (g4) for ARPE-19 cell cultures and 12.45% (e4) and 7.91% (g4) for L929 cell cultures. This demonstrated clearly that the cytotoxic effect of phenol reached to cell cultures of 4th well. Similar findings have been seen for other groups f and h. This confirmed that exposure of long period of cell cultures to phenol in an experimental plan could affect the results. The results also showed that ARPE-19 cell culture viability of groups e, f, g and h are higher than for the L929 cell cultures, confirming higher sensitive of L929 for phenol.

These results provide valuable data to demonstrate that the errors in data can be obtained if the phenol, as a positive control, is used for exposing long period to a cell culture in a cytotoxicity test method.

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