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Original Article

# Evaluation of the Cytotoxicity of Polyetherurethane (PU) Film Containing Zinc Diethyldithiocarbamate (ZDEC) on Various Cell Lines

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A polyetherurethane (PU) film containing 0.1% zinc diethyldithiocarbamate (ZDEC) is the international standard reference material for testing the *in vitro* cytotoxicity of polymer based biomaterials. Nowadays, culturing L929 or BALB/3T3 cells in direct contact or in an extract dilution condition is the most frequently using method for evaluating the cytotoxicity from biomaterials and medical devices. However, the results often vary, because it is directly related to the cellular functions and the mechanism of the toxicity of the contacting cells. In this study, 13 cell lines originating from various tissues were used to detect the cytotoxic activities of a PU film containing 0.1% ZDEC (PU-ZDEC). The correlation between the reactivity zone size and the relative cytotoxicity by quantifying the released total protein from each cell in the direct contact testing method was investigated. Hepa-1c1c7 cells demonstrated the highest sensitivity in the reactivity zone size, while CHO/dhFr(-) cells were the most sensitive in terms of the relative cytotoxicity. A correlation between the two processes in each cell line was not found (r=-0.478). In the extract dilution method, which involved cultivating the cells in the medium with various ZDEC concentrations prepared by diluting the PU incubation, the cytotoxicity increased with increasing ZDEC concentration in all cell lines. The BALB/3T3 cells demonstrated the highest sensitivity in the extract dilution method. No correlation in a comparison of the relative cytotoxicity from the direct contact method with the extract dilution method in each cell line, was found (r=-0.445). In this experiment, Hepa-1c1c7, BALB/3T3, CHO/dhFr(-) and L-929 cells among the 13 types of cell lines were the sensitive cell lines according to the two methods. The preliminary results suggest that a comparison of at least one or more cytotoxicity testing methods and many cell lines is necessary for an *in vitro* cytotoxicity test of biomaterials.

**Key Words:** Cytotoxicity, direct contact method, extract dilution method, polyetherurethane (PU) film, zinc diethyldithiocarbamate (ZDEC)

## INTRODUCTION

In recent years, the biological evaluation of biomaterials and medical devices have become more globally standardized, concurrently with the publication of the ISO 10993 standard for biomaterial and medical device testing.[1] Although an *in vitro* cytotoxicity test through a mammalian cell culture has been adopted as the primary safety evaluation method prior to an *in vivo* test in every national and international standard, the recommended guide lines for the method and cell line used vary. Accordingly, the cytotoxicity results from a specimen vary according to the standards.[2-6]

Cytotoxicity tests are recommended for all medical devices as (1) they allow a rapid evaluation, (2) employ standard protocols, (3) produce quantitative and comparable data and (4) due to their sensitivity, they allow the discarding of toxic materials prior to animal testing.[7] However, several problems have been encountered in the *in vitro* cytotoxicity testing of biomaterials, including choice of the cell type, the preparation of the test materials, the test procedure and quantification of

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the results, etc.[8]

The purpose of this study was to evaluate the cytotoxicity of 13 cell lines originating from various types of tissues on PU-ZDEC, a standard reference material (SRM) in the international guidelines for the basic biological tests of biomaterials and medical devices,[4,9,10] by the direct contact and extract dilution method.

## MATERIALS AND METHODS

### Materials

All reagents used for the cytotoxicity test were purchased from Sigma (St. Louis, Mo., USA). The PU-ZDEC (thickness 0.5 mm) and high density polyethylene (HDPE) films (thickness 0.5 mm) were purchased from Hatano Research Institute, Food and Drug Safety Center, Tokyo, Japan. The HDPE film was used as the control and PU-ZDEC was the test specimen. Both the PU-ZDEC and HDPE films ($1 \times 1 cm^2$) were sterilized using ethylene oxide gas for the cytotoxicity test.[11]

### Cells and cell culture

Thirteen different cell lines were used (Table 1). Among these cell lines, ASK and LU cell lines were supplied from the Natural Products Research Institute, Seoul National University (SNU), Seoul, Korea, and the other cell lines were purchased from the American Type Culture Collection (ATCC), Manassas, VA, USA.

All cell lines were cultured at 37℃ in a humidified atmosphere (5% $CO_2$) in six kinds of medium; (1) Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 1.0 mM sodium pyruvate, and 10% fetal bovine serum (FBS), (2) DMEM containing 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 1.0 mM sodium pyruvate, and 10% bovine calf serum (BCS), (3) Minimum essential medium Eagle (MEME) containing 2 mM L-glutamine, Earle's BBS, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acid, 1.0 mM sodium pyruvate, and 10% FBS, (4) $\alpha$-MEME without nucleosides, and containing 10% FBS, (5) RPMI-1640 medium containing 2 mM

**Table 1.** The List of Cell Lines for the Cytotoxicity Test

| Designation | Organism | Tissue | Morphology | Medium | ATCC No. |
|---|---|---|---|---|---|
| A-431 | Homo sapiens (human) | epidermis | epithelial | DMEM 10% FBS | CRL-1555 |
| AGS | Homo sapiens (human) | stomach | epithelial | RPMI-1640 10% FBS | CRL-1739 |
| ASK* | Neuroglial (rat) | brain | astrocyte | MEME 10% FBS | - |
| BALB/3T3 | Mus musculus (mouse) | embryo | fibroblast | DMEM 10% BCS | CRL-9392 |
| BHK-21 | Mesocricetus auratus (hamster) | kidney | fibroblast | DMEM 10% FBS | CCL-10 |
| CHO/dhFr(-) | Cricetulus griseus (hamster) | ovary | epithelial | IMDM 10% FBS | CRL-9096 |
| COS-7 | Cercopithecus aethiops (monkey) | kidney | fibroblast | DMEM 10% FBS | CRL-1651 |
| Hepa-1c1c7 | Mus musculus (mouse) | liver | fibroblast | $\alpha$-MEME 10% FBS | CRL-2026 |
| Hep G2 | Homo sapiens (human) | liver | epithelial | MEME 10% FBS | HB-8065 |
| HS-68 | Homo sapiens (human) | foreskin | fibroblast | DMEM 10% FBS | CRL-1635 |
| L-929 | Mus musculus (mouse) | subcutaneous connective tissue | fibroblast | DMEM 10% FBS | CCL-1 |
| LU* | Homo sapiens (human) | lung | epithelial | MEME 10% FBS | - |
| V79-4 | Cricetulus griseus (hamster) | lung | fibroblast | DMEM 10% FBS | CCL-93 |

*ASK and LU cell lines were obtained from Natural Products Research Institute, SNU, Korea.
The others cell lines were purchased from ATCC, USA.

L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% FBS, (6) Iscove's modified Dulbecco's medium (IMDM) containing 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM hypoxanthine, 0.016 mM thymidine, and 10% FBS.

**Cellular toxicity test by direct contact method**

Six-well plates were employed for the direct contact method.[12-14] The cultured cells were plated at $4.0 \times 10^5$ cells per well, and pre-incubated for 24 hours at 37℃ in a humidified atmosphere (5% $CO_2$). After 24 hours, the media was exchanged with 0.8 ml of fresh media containing 10% FBS. The specimen films ($1 \times 1$ $cm^2$) were over laid on the center of the well where a confluent monolayer of the cells formed, and incubated for 24 hours. After removing the specimens from each well, the well was washed with PBS and stained with 0.2% crystal violet (CV) in a 2% ethanol (EtOH) solution for 20 minutes. While the normal cells in the areas unaffected by specimens were well stained by the CV, the dead cells were detached from the well plate during the staining process. The detached area affected by cytotoxic specimen was transparent, and could therefore, be easily discriminated from the unaffected and sufficiently stained area. The results were compared with the controls, and interpreted by the grade of the reactivity zone (Table 2). After measuring the zone size, the stained cells were lysed in a 0.5% sodium dodecyl sulfate (SDS) solution in 50% EtOH, and transferred to a 96-well plate. The absorbance of each well was measured at 610 nm using an automatic microplate reader (Spectra Max 340, Molecular Device Inc., Sunnyvale, CA., USA). The cytotoxic potential of the PU-ZDEC film on each cell line was expressed in terms of the relative cytotoxicity with regard to absorbance of the control well as 100%, and the percentage of the absorbance of each well was calculated.

**ZDEC-realease assay**

Prior to the extract dilution method, the concentration of the released ZDEC from the incubated specimen was investigated in order to obtain optimal extraction time. Each specimen ($1 \times 1$ $cm^2$) was placed into a screw capped bottle containing phosphate buffered saline (PBS) solution, and agitated at 100 rpm for 24, 48 and 72 hours at 37℃ to provide a dynamic condition for ingredient release from the specimens. After shaking, the absorbance of the extracted PBS solution was measured at 280 nm by an ultra violet visible spectrophotometer (UV-1601PC, Shimadzu, Japan).

**Cellular toxicity test by extract dilution method**

For the extract dilution test, the modified CV staining method was performed as described by Lee et al.[15] The extracts from the specimens were prepared by shaking at 100 rpm for 48 hours at 37℃. The extracts were then serially diluted 2-fold by adding fresh media containing 10% FBS. The cells were seeded at a density of $2 \times 10^5$ cells/well into 24-well plate in duplicate, and incubated at 37℃ for 24 hours. The medium was replaced with diluted extract media and incubated for a further 24 hours. Each well was then washed by PBS and stained with 0.2% CV in a 2% EtOH solution. The stained cells were lysed by 0.5% SDS solution in 50% EtOH, and transferred into a 96-well plate. The absorbance of each well was measured at 610 nm by an automatic microplate reader. The cytotoxic potential of PU-ZDEC on each cell line is expressed in terms of the relative

**Table 2.** The Reactivity Grades for the Direct Contact Test*

| Grade | Reactivity | Description of reactivity zone |
|---|---|---|
| 0 | None | No detectable zone around or under specimen |
| 1 | Slight | Zone limited to area under specimen |
| 2 | Mild | Zone extends less than 0.5 cm beyond specimen |
| 3 | Moderate | Zone extends 0.5-1.0 cm beyond specimen |
| 4 | Severe | Zone extends greater than 1.0 cm beyond specimen but does not involve entire dish |

*From ASTM standards, Vol. 13.01[13].

cytotoxicity and the IC50. The IC50 (× $\chi$; this sign expresses dilution factors), which is the dilution factor of an extract from a test material inhibiting the growth of cells by 50%, was estimated from the cytotoxicity.

**Statistical analysis**

The data is expressed as a mean ± SEM of duplicate. Fisher's correlation coefficient r was calculated.

## RESULTS

**ZDEC-release test**

The concentration of the released ZDEC from the PU-ZDEC film showed a maximum value at 48 (4.09 ± 0.56 $\mu$g/ml) to 72 hours (4.33 ± 0.2 $\mu$g/ml). Based on this result, the extraction time of the PU-ZDEC for preparing the test samples in the extract dilution method was determined to be 48 hours (Fig. 1).

**Cellular toxicity test by direct contact and extract dilution method**

The cytotoxicity of PU-ZDEC was evaluated by both the direct contact and extract dilution method using 13 cell lines. The results from the two methods were compared. In the direct contact test, the size of the reactivity zone in each cell lines was observed. The cells cultured under and around the PU-ZDEC were detached from the culture dish and produced a round shaped cell-less zone on the plate by the released ZDEC from the PU-ZDEC film. Among the test cell lines, although the L-929, BALB/3T3, HS-68, CHO/dhFr(-) and Hep G2 cells exhibited a relatively high sensitivity to the cytotoxicity of PU-ZDEC, Hepa-1c1c7 was found to be the most sensitive cell line. The order of the sensitivity was: Hepa-1c1c7 > L-929 > BALB/3T3 > HS-68 > CHO/ dhFr(-) > Hep G2 > ASK > AGS > BHK-21 > A-431 > COS-7 > LU > V79-4 cells (Table 3).

In contrast, CHO/dhFr(-) was found to be the most sensitive cell line to the cytotoxicity of PU-ZDEC after lysing the CV stained cells with 0.5% SDS solution, and Hepa-1c1c7, L-929 and BALB/3T3 also demonstrated a comparatively high sensitivity. The order of the relative cytotoxicity was: CHO/dhFr(-) (50.24%) > Hepa-1c1c7 (43.73%) > L-929 (36.59%) > BALB/3T3 (32.5%) > AGS (30.8%) > Hep G2 (28.51%) > V79-4 (27.97%) > LU (27.81%) > COS-7 (26.15%) > ASK (25.41%) > A-431 (24.48%) > BHK-21 (16.74%) > HS-68 (10.61%) cells (Fig. 2).

In order to determine the correlation between the conventional size detection and the relative

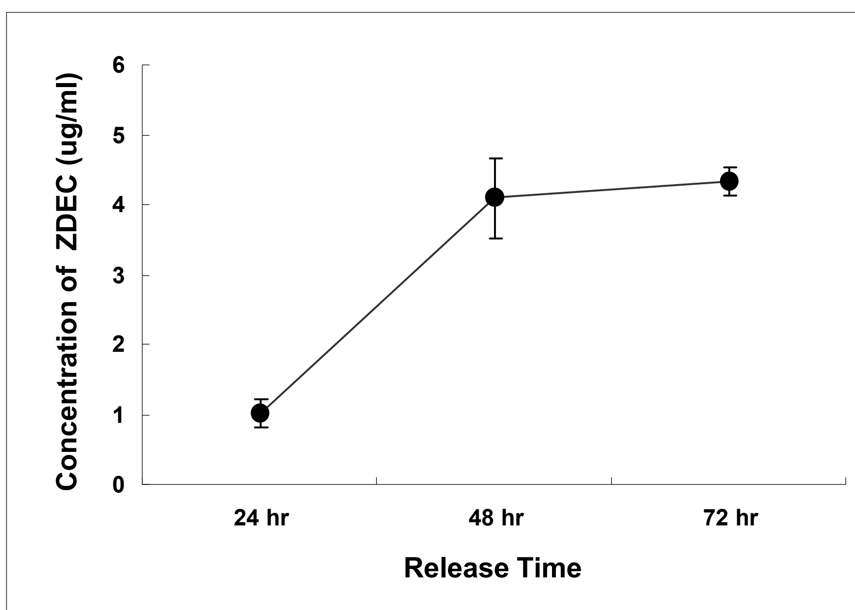


**Fig. 1.** The concentration of the released ZDEC from the PU film containing 0.1 % ZDEC. The ZDEC was released from the PU-ZDEC film (1 × 1 cm$^2$) by shaking at 100 rpm for 24, 48 and 72 hours at 37℃. After shaking, the absorbance of the extraction media was measured at 280 nm by an UV-visible spectrophotometer.

**Table 3.** The Grade of Reactivity Zone of PU-ZDEC Film in the Direct Contact Method

| Cell lines | PU film containing 0.1 % ZDEC | |
|---|---|---|
| | Formed zone size (cm) | Zone index |
| A-431 | 0.44 ± 0.04 | 2 |
| AGS | 0.48 ± 0.07 | 2 |
| ASK | 0.49 ± 0.01 | 2 |
| BALB/3T3 | 0.60 ± 0.02 | 3 |
| BHK-21 | 0.48 ± 0.02 | 2 |
| CHO/dhFr(-) | 0.57 ± 0.04 | 3 |
| COS-7 | 0.43 ± 0.02 | 2 |
| Hepa-1c1c7 | 0.80 ± 0.05 | 3 |
| Hep G2 | 0.53 ± 0.02 | 3 |
| HS-68 | 0.58 ± 0.08 | 3 |
| L-929 | 0.64 ± 0.04 | 3 |
| LU | 0.43 ± 0.02 | 2 |
| V79-4 | 0.38 ± 0.02 | 2 |

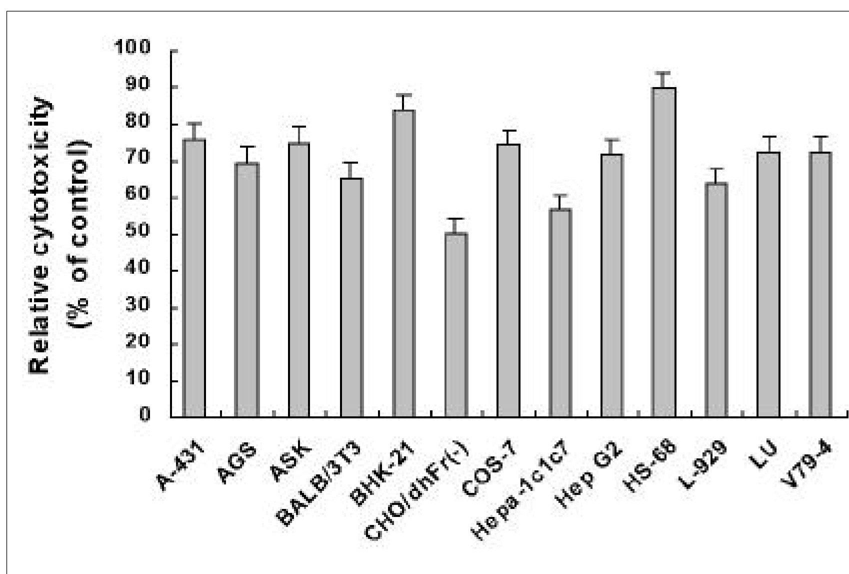


**Fig. 2.** The relative cytotoxicity of the PU-ZDEC film by 13 cell lines in the direct contact method. After measuring the zone size, the stained cells were lysed with a 0.5% SDS solution in 50% EtOH and transferred to a 96-well plate. The absorbance of each well was measured at 610 nm by an automatic microplate reader. In order to calculate the relative cytotoxicity, the absorbance of the control well was regarded as 100%, and the percentage of the absorbance of each well was calculated.

sensitivity in the direct contact method, the correlation coefficients were calculated by a correlation analyses, and a poor correlation between reactivity zone size and relative cytotoxicity was found (r=-0.478) (Fig. 3).

In the extract dilution test, all the cell lines demonstrated a dose-dependent relationship between the cytotoxic potential of a specimen and the ZDEC concentration. In particular, among these cell lines, BALB/3T3 was found to be the most sensitive cell line, and Hepa-1c1c7 and V79-4 were also more sensitive than the others. The order of relative cytotoxicity in the extract dilution test was: BALB/3T3 (IC50: × 4.3) > Hepa-1c1c7

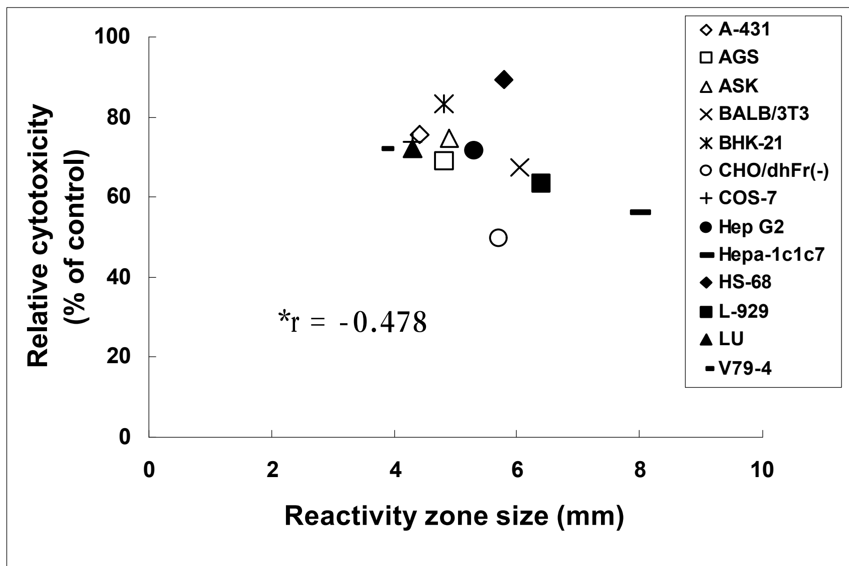


**Fig. 3.** The correlation between the reactivity zone size and the relative cytotoxicity in direct contact method. The coefficient of correlation was calculated by correlation analyses, and no correlation between the two processes was found in each cell line (r=-0.478).

(IC50: × 4.2) > V79-4 (IC50: × 3.5) > AGS (IC50: × 2.9) > COS-7 (IC50: × 2.6) > L-929 (IC50: ×2.4) > BHK-21 (IC50: × 2.4) > Hep G2 (IC50: ×2.2) > ASK (IC50: × 2.1) > CHO/dhFr(-) (IC50:× 2.1) > A-431 (IC50: × 1.0) > HS-68 (IC50: × 1.0) > LU (IC50: × 0.7) cells (Fig. 4).

In order to investigate the correlation between the direct contact and the extract dilution method, the coefficient of correlation was calculated. However, there is no significant correlation (r=-0.445) between these two methods.

## DISCUSSION

Cytotoxicity testing is a useful tool for evaluating biomaterials and medical devices, and there are three types of cytotoxicity tests generally used: the extract method, the direct contact method, and the indirect contact test.[6]

In this study, the extract method and the direct contact method, were used to evaluate the cytotoxicity PU-ZDEC on 13 cell lines, which have quite different characteristics such as morphology and specific functions, originating from various tissues. This is because, in part 5 (Tests for cytotoxicity: *in vitro* methods) of the ISO guidelines for assessing the biological compatibility of medical devices (ISO 10993), it was reported that ... no single method is able to detect the cytotoxicity of devices. *per se*..'. This means that the ISO 10993-5 recommended that one or more evaluation methods should be used to test the cytotoxic effect of biomaterials and medical devices.[12]

The direct contact method and another extract dilution method are more commonly adopted for the cytotoxicity evaluation in the general *in vitro* testing guidelines for materials and devices using in the body.[11-13] The direct contact method enables weak cytotoxicity to be detected because of its high sensitivity,[8,16] and the extract dilution method is commonly used since it can be applied to a wide variety of raw materials and finished products that release the extracts from all exposed surfaces.[11-13,15]

The direct contact method has various disadvantages; (i) cellular trauma if the material moves, (ii) cellular trauma with high density materials, (iii) reduced cell population with highly soluble toxic agents.[17] From the results of the direct contact test, as shown in Table 3, Hepa-1c1c7 was found to be the most sensitive cell line in the reactivity zone size, whereas in terms of the relative cytotoxicity, CHO/dhFr(-) was found to be the most sensitive cell line in comparison with the others, as shown in Fig. 2. There was no correlation between the reactivity zone size and the relative cytotoxicity (r=-0.478). These results suggest that there is a difference in the cellular resistance against the external mechanical loads, which was oriented by placing the PU-ZDEC specimen directly on the cells. Hepa-1c1c7 cells,

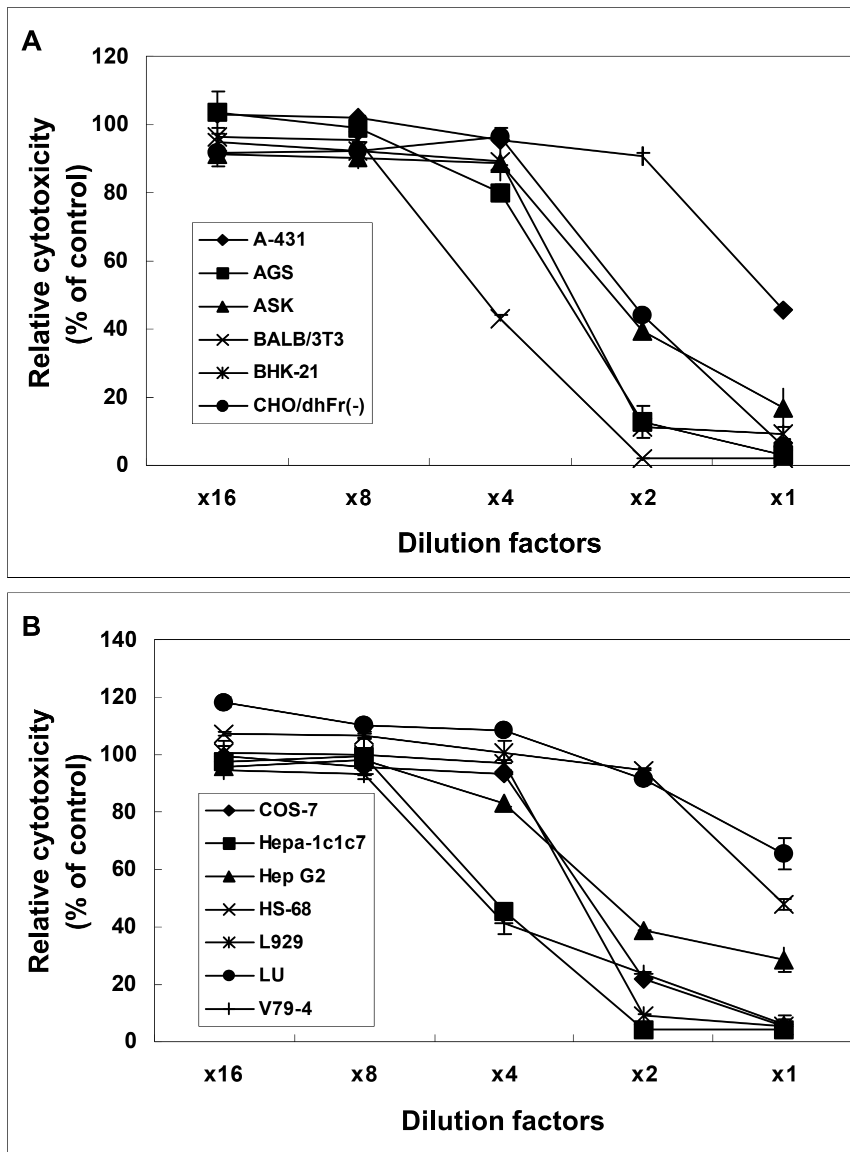


**Fig. 4.** The relative cytotoxicity of PU-ZDEC using 13 cell lines originating from various types of tissues in the extract dilution method (A and B). The extracts of the specimens were prepared by shaking at 100 rpm for 48 hours at 37℃, and serially diluted by adding fresh media containing 10% FBS. The cells were seeded at a density of 2.0 $\times 10^5$ cells/well into 24-well plate in duplicate, and incubated at 37℃ for 24 hours. The medium was replaced by the extract or its diluted extract and incubated for another 24 hours. Each well was then washed with PBS and stained with 0.2% CV in 2% EtOH solution. The stained cells were lysed with 0.5% SDS solution in 50% EtOH and transferred to a 96-well plate. The absorbance of each well was measured at 610 nm. In order to calculate the relative cytotoxicity, the absorbance of the control well was regarded as 100%, and the percentage of the absorbance of each well was calculated.

which are from the liver and are not normally exposed to the mechanical stress, revealed the highest sensitivity in the reactivity zone size. LU and V79-4, which are from the lungs and are continuously exposed to a physiological load, demonstrated less sensitivity. In addition, the difference in the proliferative potential of each cell line might also affect the sensitivity. This is because, the life cycle of each cell line is different. Therefore, a comparison of the reactivity zone size with the relative cytotoxicity activity of biomaterials and medical devices is needed in order to avoid possible errors in interpreting the experimental results.

Furthermore, in testing the cytotoxicity, the crucial parameters are the types of cells, the duration of the exposure, the physical forms of the devices and the methods of evaluation.[7] Among these parameters, the types of cells could be the most important factor, because each cell's function and toxicity mechanism is basically different. For these reason, the recommended cell lines for cytotoxicity testing in the ISO 10993-5 are as follows; L-929, BALB/3T3, MRC-5, WI-38, Vero, BHK-21, and V79 cells.[12]

In this study using thirteen cell lines originating from various types of tissues including the recommended cell lines, the results from the extract

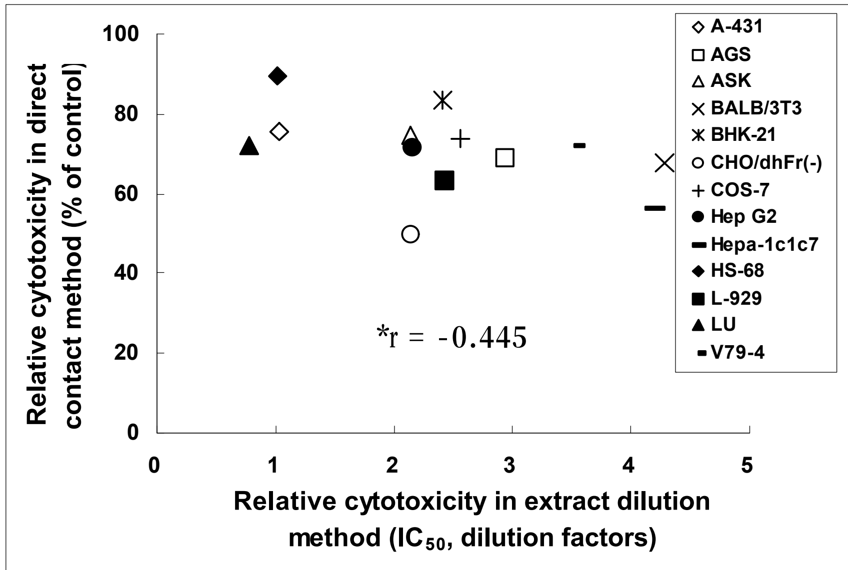


**Fig. 5.** The correlation of the cytotoxicity between the direct contact and the extract dilution method. No correlation was found (r=-0.445).

dilution method showed a dose-dependent relationship and a variable cytotoxic potential that was related to the types of cell lines, as shown in Fig. 4. Among these cell lines, BALB/3T3 and Hepa-1c1c7 cells were found to be more sensitive cell lines than others. In contrast, Tsuchiya T. et al.[4,9,10,15] used only BALB/3T3, L-929, and V-79 cells for testing the cytotoxicity of PU-ZDEC, and reported that the order of relative cytotoxicity in the extract dilution test was: BALB/3T3 > L-929 > V79-4. This results are different from those in this study. This difference suggests that the types of cells are very important for testing the cytotoxicity of biomaterials and medical devices such as catheters, contact lens, orthopedic plates, and healing devices.

In order to compare the direct contact and extract dilution method, the correlation coefficient was calculated by correlation analyses. Fig. 5 shows there was no correlation (r=-0.445). This suggests that different cell types and evaluation methods have different sensitivities to the same biomaterials and medical devices, and the methods of evaluation and the types of cells are the crucial parameters. Therefore, the selection of the evaluation methods and the cell lines are an important factor for testing the cytotoxic effect.

In conclusion, Hepa-1c1c7, BALB/3T3, CHO/dhFr(-) and L-929 cells were the most sensitive cell lines to PU-ZDEC of the 13 cell lines using the two methods. The preliminary data suggests that a concurrent combination of two or more evaluation methods and specific cell lines in accordance with the test materials are required for *in vitro* cytotoxicity testing.

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