

Evaluation of the Viability of L-929 Cells in the Presence of Alendronate and Absorption Enhancers

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Summary

Alendronate and two absorption enhancers (dimethyl- β -cyclodextrin and sodium taurocholate) were selected as model substances. The cytotoxicity of alendronate and the absorption enhancers was evaluated on mouse fibroblast cells (L-929) by using MTT assay. Higher concentrations of alendronate and the absorption enhancers demonstrated greater toxicity for L-929 cells. Serum presence in culture medium decreased the toxicity potential. Longer period of exposure of the cells to the substances as a consequence of extended experiment period also decreased the cell viability.

Key Words: Alendronate, sodium taurocholate, dimethyl- β -cyclodextrin, L-929, MTT test.

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L-929 Hücrelerinin Canlılığının Alendronat ve Absorpsiyon Artırıcılar Varlığında Değerlendirilmesi

Özet

Alendronat ve iki absorpsiyon artırıcı (dimetil- β -siklodekstrin ve sodyum taurokolat) model maddeler olarak seçilmiştir. Alendronat ve absorpsiyon artırıcıların sitotoksitesi fare fibroblast hücreleri (L-929) üzerinde MTT analiz yöntemi ile değerlendirilmiştir. Alendronat ve absorpsiyon artırıcıların yüksek konsantrasyonlarının L-929 hücreleri için daha fazla toksik oldukları gözlenmiştir. Kültür ortamındaki serum varlığı toksite potansiyelini azaltmıştır. Maddelerin hücreler ile temas süresinin uzunluğu L-929 hücrelerinin canlılığını azaltmıştır.

Anahtar kelimeler: Alendronat, sodyum taurokolat, dimetil- β -siklodekstrin, L-929, MTT testi

INTRODUCTION

Alendronate is a nitrogen-containing bisphosphonate¹ being used in the prevention and treatment of bone diseases². It reduces the risk of bone fractures by increasing the mineral density, mineralization period and strength of bone³.

Alendronate is generally well-tolerated after a short- or long-term usage³, but esophagitis and gastric side effects have also been reported^{4,5}. Many endoscopic studies show that alendronate is toxic in the upper gastrointestinal tract^{6,7}. Dimethyl- β -cyclodextrin (DM- β -CD) and sodium taurocholate (STC) are the absorption enhancers being used in many formulations.

Before starting a cell culture transport test or in vivo experiments, it is essential to carry out preliminary in vitro tests to screen and characterize the potentially harmful effects of a material to the tissues. The concentration-dependent cytotoxicity of the substances can be determined by cytotoxicity tests. Tetrazolium salts have been used extensively to demonstrate the reductive capacity of tissues⁸, and a quantitative microtiter assay for cell proliferation and cytotoxicity⁹. This assay is now in widespread use to measure viable cells with particular applications in drug screening programs¹⁰. Reduction of MTT by succinic dehydrogenases in living cells is the basis of this mitochondrial viability assay¹¹.

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It is critical to select the appropriate cell types for cytotoxicity assays. International Standard (ISO 10993 part 5, 1999) prefers the use of established cell lines such as L-929, Balb/3T₃ and WI-38 for cytotoxicity testing. These cell lines provide good reproducibility for in vitro cytotoxicity screening owing to their homogeneous morphology and growth characteristics. The L-929 cell line is a mouse fibroblast cell. To compare, a series of cytotoxicity tests using the L-929 cell line were performed based on references from International Standards (ISO 10993 part 5, 1999; ISO 7405, 1997) and INVITTOX protocols (31; November 1991)¹².

The aim of this study was to determine the cytotoxic effects of alendronate, DM-β-CD and STC on L-929 cells. During the experimentation, the effects of serum presence in culture medium and length of experiment period on the viability of L-929 cells were also investigated.

MATERIALS and METHODS

Materials

Alendronate sodium trihydrate was kindly donated from Sanovel Pharmaceutical Company (Turkey). DM-β-CD and STC were purchased from Cyclo Laboratory (Hungary) and Sigma (Germany), respectively. DMEM, medium components and other reagents for cell culture were obtained from Gibco Life Technologies (England). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was purchased from Sigma (Germany). All other materials were of analytical grade.

Method

L-929 cell culture

L-929 cells originating from mouse fibroblast cells were obtained from American Type Culture Collection (ATCC) and grown by the Food and Mouth Diseases Institute (Turkey). Cells which were kept at -196°C were dissolved in water bath at 37°C, then centrifuged at 800 rpm for 5 min. The precipitant was homogenized with DMEM supplemented with 10% heat-

inactivated fetal calf serum, 1% sodium bicarbonate, 1% gentamicin and then grown in 25 cm² cell culture flasks (Costar-Germany). Flasks were kept at 37°C and the medium was changed every 48 h until the flasks reached 100% confluence. Cells were passaged and the volume was enlarged to 75 cm². Cells were detached from the flasks by incubating the monolayers with trypsin EDTA. Cells were collected and counted with a hemocytometer, and then were seeded in 96-well culture plates (Cellstar-Greinerbio-one) for cytotoxicity studies.

Cytotoxicity studies

Cell viability studies in the presence of alendronate and the absorption enhancers were performed via MTT test. MTT tests were conducted by applying DMEM supplemented with and without serum, for 8 and 24 h periods. L-929 cells were resuspended in DMEM and plated into the microwells of 96-well tissue culture plates. Plates were incubated for 24 h at 37°C in a humidified incubator containing 5%CO₂/95%O₂ and then the medium was removed from wells. 100 μL DMEM containing test solutions (alendronate, DM-β-CD, STC) (Table 1) were added into the wells. After 8 or 24 h, the medium containing the drug or the absorption enhancers was removed. Each well was treated with 100 μL medium and 13 μL MTT solution, and incubated for a further 3 h. Then, plates were emptied and 100 μL isopropanol was added to dissolve the formazan precipitate. The developed color was read at a wavelength of 570 nm with spectrophotometer (Versamax Molecular Devices).

Table 1. Concentrations of samples used in cytotoxicity tests

Samples	Concentrations in DMEM (8 h period)	Concentrations in DMEM (24 h period)
Alendronate	0.1, 0.2, 0.4, 0.8, 1.2, 1.6	0.1, 0.2, 0.4
DM-β-CD	0.3, 0.6, 1.2, 2.5, 5	0.3, 0.6, 1.2
STC	1.2, 2.5, 5, 10, 20	1.2, 2.5, 5

Statistical analysis

Cytotoxicity test data was analyzed using two-sided Student's t test, and a value of p<0.05 was considered significant.

RESULTS and DISCUSSION

A detailed literature search shows that L-929 cells are being widely used for cytotoxicity analysis^{11,12}. However, we could not find any data concerning the use of L-929 cells in cytotoxicity testing for alendronate or absorption enhancers. Different cell lines like Caco-2 have been used to investigate the cytotoxicity of different bisphosphonates¹³⁻¹⁶, but there is no study about alendronate. International Standard prefers the use of L-929, Balb/3T₃ and WI-38 for cytotoxicity studies¹¹; therefore, L-929 cells were used in this study. Caco-2 cells were also used to determine the toxicity of alendronate and absorption enhancers, and it was found that Caco-2 cells were generally more resistant for the investigated compounds than L-929 cells¹⁷⁻²⁰.

The dose-dependent viability of L-929 cells treated with alendronate is presented in Figure 1. The cytotoxic effect of alendronate on L-929 cell viability changed according to the experiment period. Cell viability was decreased in the 24 h period when compared with 8 h. The viability of L-929 cells in the 8 h incubation period in the presence of serum was not significantly different from that obtained in the absence of serum ($p=0.4937$, >0.05). However, viability of L-929 cells in the 24 h incubation period in the presence of serum was significantly higher than that observed in the absence of serum ($p=0.0326$, <0.05).

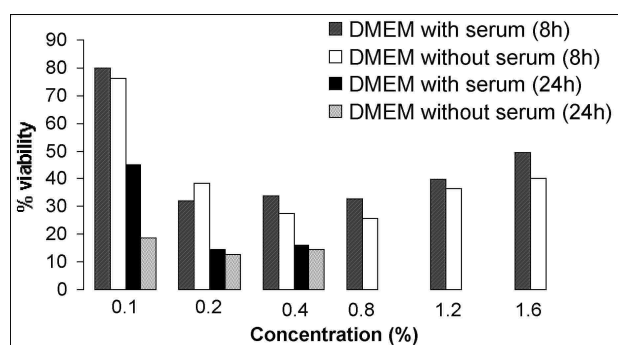


Figure 1. □ MTT assays on L-929 cells for different concentrations of alendronate.

The concentration of 0.6% seemed to be critical for DM- β -CD (Fig. 2). A minimum plateau was reached at about 0.6%. The viability of L-929 cells decreased up to this concentration as a consequence of cell death.

As observed for alendronate, serum-present DMEM and 8 h period decreased the cell viability less than 24 h period and serum-free DMEM. The viability of L-929 cells in the 8 h period in the presence and absence of serum was found to be significantly different ($p=0.0342$, <0.05) whereas the effect of serum on viability in 24 h was not significant ($p=0.0831$, >0.05).

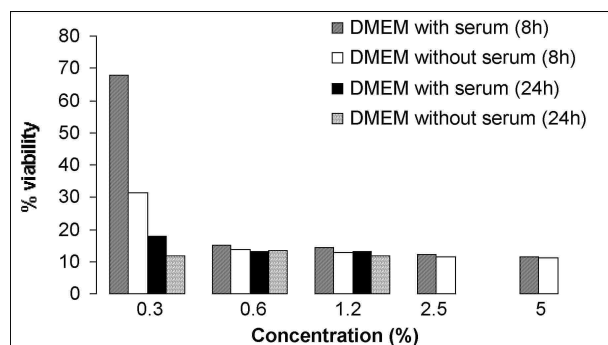


Figure 2. □ MTT assays on L-929 cells for different concentrations of DM- β -CD.

According to MTT test for STC (Fig. 3), difference in the viability of L-929 cells in the 8 h period in the presence and absence of serum was not statistically significant ($p=0.2817$, >0.05). The 24 h period was more toxic at the concentration of 5 mM. We can consider that cells are not viable above a concentration of 10 mM.

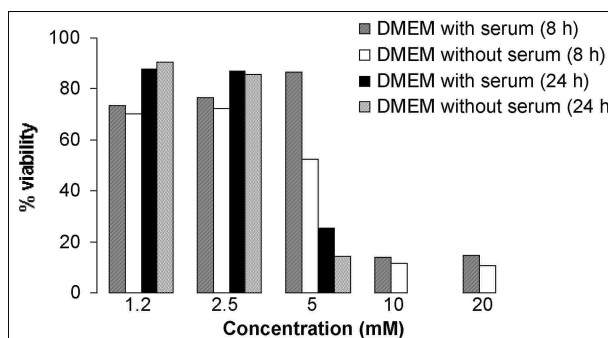


Figure 3. □ MTT assays on L-929 cells for different concentrations of STC.

The viabilities at selected concentrations in the presence of serum were slightly higher than those determined without serum (Figs. 1, 2, 3), so we can suggest that serum existence in culture medium can also affect the viability of cells.

During the MTT test, we sometimes observed increases in viability which could be attributed to the increase in tetrazolium salt transported inside the cells^{13,16}.

CONCLUSION

L-929 cells can be used for determining the toxic concentrations of substances. Higher concentrations of drugs or pharmaceutical excipients can decrease the viability of cells so it is important to choose the most convenient concentration that is not toxic to cells. The experiment period or additional experimental conditions, such as serum existence, should be taken into consideration when performing the experiments. Comparing the histopathologic data of L-929 cells with MTT results can also provide more information about the cell damage.

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