The viability of cells exposed to material extracts was assessed by measuring succinic dehydrogenase activity. The succinic dehydrogenase activity has been shown to be reasonably representative of mitochondrial activity in the cells and reflects both cell number and activity.[11] The old medium was removed and cell cultures were rinsed with sterile phosphate-buffered saline and 0.5 ml of a freshly prepared MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) dye solution (Sigma, Taufkirchen, Germany) (0.5 mg/ml in BME) were added to each well. After incubation for 2 h (at 37°C, with 5% $CO_2$), the supernatant was removed and the intracellularly stored MTT formazan was solubilized in 200 µL dimethyl sulfoxide for 30 min at room temperature. The spectrophotometric absorbance was measured at 540 nm using a spectrophotometer (µQuant, Bio-Tek Instruments, Winooski, VT, USA). Twelve wells were used for each specimen. Testing was repeated twice to ensure reproducibility.

### Statistical analysis

The one-way analysis of variance test was used to determine variance for each sealer and control group. The significant differences between groups were analyzed using the Tukey-HSD procedure, with the value of statistical significant being set at $P < 0.05$. All computations were made using Statistical Package for the Social Sciences (SPSS) 10.0 statistical software (SPSS Inc., Chicago, IL, USA).

## RESULTS

The results of the MTT assays are given in Table 2 for the L929 and Saos-2 cells. Results with both L929 fibroblasts and Saos-2 cells demonstrated that all tested sealers reduced vital cell number in comparison with the control group ($P < 0.05$) [Figures 1 and 2]. For L929 cells revealed that cell survival with extracts of EndoREZ, Epiphany SE, EZ-Fill, MMSeal and AHPlus was 12.1 ± 0.9%, 46.2 ± 4.1%, 12.0 ± 0.8%, 44.0 ± 5.5% and 12.4 ± 1.1%, respectively. The ranking of the most to the least toxic material was: EZ-Fill = EndoREZ = AHPlus >> MMSeal = Epiphany SE. For Saos-2 cells revealed that cell survival with extracts of EndoREZ, Epiphany SE, EZ-Fill, MMSeal and AHPlus were 33.9 ± 3.8%, 32.9 ± 3.2 %, 33.1 ± 5.6 %, 35.3 ± 3.9 % and 34.6 ± 4.2 %, respectively. For Saos-2 cells, all tested sealers showed moderate cytotoxicity [Table 2].

## DISCUSSION

In this study, the MTT assay was used to evaluate the cytotoxicity of five different resin-based sealers. MTT is a water-soluble, tetrazolium salt yielding a yellowish solution when prepared in media or salt solution. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes in living cells. The test results reflect not only the cell number, but also the vital cell metabolic level. However, different methodologies or cell lines may affect the results, which can create problems in comparing the data from different sources.[8,12] Therefore, a set of standardized-assay procedures was established and used for all test materials evaluated in this study so that the results are comparable.

Either established permanent cell lines, such as L929 cells or primary cells can be used to test cytotoxicity of dental materials when employing *in vitro* methods of experimentation. The advantage of permanent cell lines is they will continue to grow as long as sustenance is available for them. Primary cells have a predetermined life span and will eventually reach a plateau of growth and then die, even if the conditions for growth are acceptable.[6] The criteria for evaluating the clinical success and failure of endodontic treatment rely heavily on radiographic interpretation of bone density.[13] In addition, because the materials tested would more likely come into contact with human bone cells *in vivo*, evaluation of the response of bone cells to filling materials is important. In this study, in order to more closely represent clinical conditions for cytotoxicity

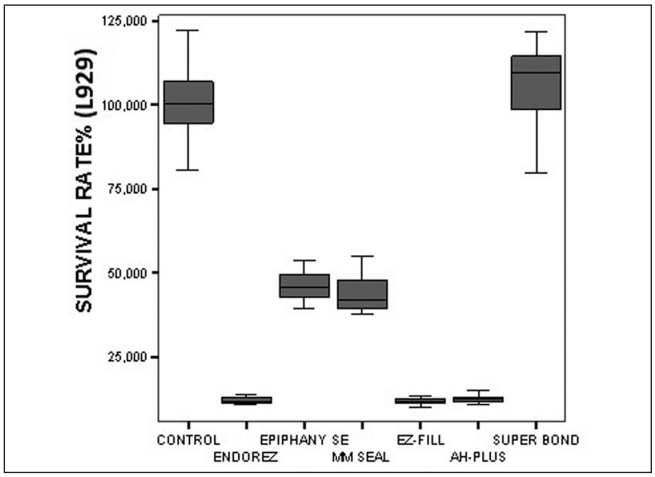


**Figure 1:** Cytotoxic effects of five different resin-based sealers on mouse skin fibroblast cells by methylthiazole tetrazolium assay. Percentage of absorbance at each elute was calculated and compared with that of control

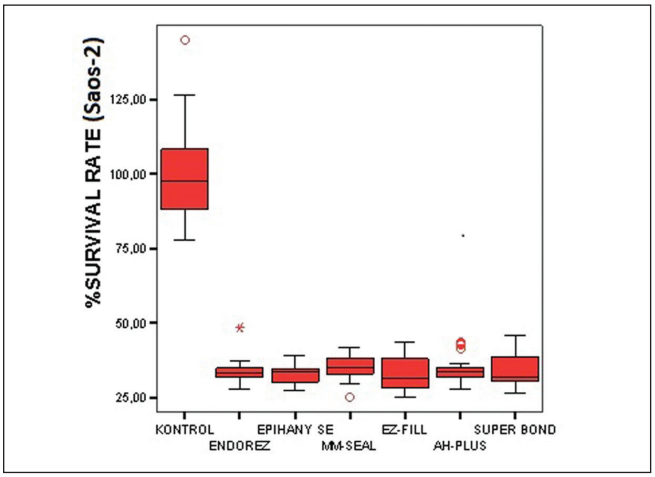


**Figure 2:** Cytotoxic effects of five different resin-based sealers on sarcoma osteogenic-2 cells by methylthiazole tetrazolium assay. Percentage of absorbance at each elute was calculated and compared with that of control

**Table 2: Cytotoxic effect of five different resin based root canal sealers on L929 and Saos-2 cells expressed in percentage of viable cells compared with control in freshly samples. The rating of cytotoxicity for each sealer according to cell type indicated in the last columns**

| Cell viability % (mean±SD) (n=24) | | |
|---|---|---|
| **Sealer** | **L929 cells** | **Saos-2 cells** |
| EndoREZ | 12.1±0.9[b] | 33.9±3.8[a] |
| EpiphanySE | 46.2±4.1[a] | 32.9±3.2[a] |
| EZ-fill | 12.0±0.8[b] | 33.1±5.6[a] |
| MMSeal | 44.0±5.5[a] | 35.3±3.9[a] |
| AHPlus | 12.4±1.1[b] | 34.6±4.2[a] |

Within each set of columns, same superscript letters indicate that do not differ significantly (ANOVA, Tukey-HSD, $\alpha$=0.05), SD: Standard deviation

evaluation of sealers, human bone osteosarcoma cells (Saos-2) were used, together with the mouse skin fibroblasts (L929) commonly used in cytotoxicity evaluations.

Determining both short- and long-term cytotoxicity of sealers might be important. The short-term toxicity of sealers may induce milder *in vivo* inflammatory responses in the periradicular area, which in turn may cause less postobturation symptoms, such as swelling and pain. Furthermore, the healing process likely may occur earlier, compared to conditions in which the cytotoxicity of a sealer lasted for longer periods of time.[14] However, a common finding observed with the traditional sealers is that sealer toxicity is significantly reduced or even eliminated after setting.[15-20] Overextended sealers represent chemical irritation as virtually all endodontic sealers are highly toxic when freshly prepared.[15] The cytotoxicity testing of freshly mixed sealers is clinically relevant as they are introduced into canals in the unset state. Huang *et al.*[21] stated that the difference in toxicity patterns at the various elution times for different sealers may be related to the degree of setting. Therefore, a sealer with a long setting time may show longer periods of cytotoxic effect. Consequently, early cytotoxic effect of the sealers seems more important than late cytotoxic effect. In the presented study, only freshly prepared sealers were used to investigate early cytotoxic effect by simulating the common clinical condition in which the sealer is extruded out of the space during canal filling.

The percentage of viable cells represents the level of cytotoxicity of the test materials. In this study, to determine the cytotoxicity, we compared the number of viable cells with the control cells. The choice of these five resin-based sealers was based on their currently increasing popularity and on the manufacturers' indications of their low toxicity. Our results indicate that although toxicity varied according to cell lines and sealers, immediately after mixing, all sealers used in the study had a cytotoxic effect. For L929 cells, EndoREZ, EZ-Fill, and AHPlus were strongly cytotoxic, whereas Epiphany SE and MMSeal were moderately cytotoxic. For Saos-2 cells, all tested sealers showed moderate cytotoxicity. The different response between L929 and Saos-2 cells to various sealers is difficult to explain and is probably due to differences in the origin of cells. Thus, we may recommend using different permanent cell lines and/or primary cells for screening the cytotoxic effects of sealers.

AHPlus is a two-component paste sealer, based on polymerization reaction of epoxy resin-amines. In the studies by Merdad *et al.*[22] and Lodiene *et al.*,[23] AHPlus showed cytotoxic effect immediately after mixing, but none or an undetectable amount 24 h after mixing. In a study by Azar *et al.*,[14] the freshly mixed AHPlus was cytotoxic, but its initial cytotoxicity was undetectable after 4 h. The short-term cytotoxicity of AHPlus has been attributed to release of formaldehyde[24] and to a lesser extent, to amines added to accelerate the polymerization reaction.[24,25]

EZ-Fill is composed of powder and gel and it has a hydrophobic nature and epoxy resin chemistry. Cohen *et al.*[26] showed that for AHPlus and EZ-Fill the amounts of formaldehyde release are 3.9 and 540 ppm, respectively. Therefore, cytotoxicity of a sealer might be not being based only on formaldehyde release because both sealers showed strong cytotoxic effect for L929 cells while they showed moderate cytotocity for Saos-2 in the present study. Bisphenol a diglycidyl ether was identified as a mutagenic component of resin-based materials, which may also be cytotoxic.[27,28] The gel component of EZ-Fill contains bisphenol a diglycidyl ether, according to the manufacturer. Therefore, bisphenol a diglycidyl ether also could be particularly responsible for EZ-Fill's cytotoxic effect.

EndoREZ is a hydrophilic, two-component, chemical-set material containing zinc oxide, barium sulfate, resins and pigments in a matrix of urethane dimethacrylate (UDMA) resin. In the present study, EndoREZ showed a strong toxic effect on L929 cells while it showed moderate cytotoxic effect on Saos-2 cells. In an *in vitro* study, Bouillaguet *et al.*[29] reported that EndoREZ presented significant cytotoxic risks when freshly mixed to Balb/c 3T3 mouse fibroblasts. In an animal study, subcutaneous implantation of EndoREZ in the connective tissue of rats caused mild to severe tissue reactions that subsided after 30 days. Also, Zmener[30] demonstrated that after subcutaneous implantation of fresh EndoREZ, components such as zinc and barium were present in tissues in direct contact with the sealer. Some of the early studies reported various degrees of toxicity from different concentrations of zinc and barium.[31,32] Also, in an *in vitro* study, Reichl *et al.* stated that the following range of increased toxicity was found: Hydroxyethyl-methacrylate (HEMA) < Triethyleneglycoldimethacrylate (TEGDMA) < UDMA < Bisphenolglycidyldimethacrylate (BisGMA). Therefore, UDMA in the EndoREZ structure, together with components such as zinc and barium, also could be responsible for the cytotoxic effect.[33]

Epiphany SE is a new version of the resin-based sealer Epiphany, in which the priming step is eliminated. Epiphany SE, containing a variety of compounds [Table 1], exhibited moderately cytotoxic potential to both cell lines. Chang *et al.*[34] have shown that HEMA alone can suppress cellular growth and cell cycle progression. Therefore, this result can be due to UDMA, HEMA, and BisGMA components in the structure of Epiphany SE. The toxicity of Epiphany has been also revealed in previous studies.[6,23,35]

The new resin sealer MMSeal has been developed recently. Our study showed that MMSeal has a moderately toxic potential to both cell lines. No published data is available about *in vitro* cytotoxicity of this sealer. Therefore, validation and extension of our results await further investigation.

Based on the results obtained from the present study, all tested resin-based sealers appear to have toxic potential to both cells in spite of different toxicity degree. Previous studies have reported a variety of cytotoxicity for these materials. Our results are in agreement with results of Ashraf *et al.*[36] and Silva *et al.*[37] who have reported that ah plus, epiphany and endorez exhibit similar toxicity in relation to cell survival. Therefore, manufacturers need to develop better sealers with acceptable biological properties for filling. In addition, because the results of *in vitro* assays may not be directly comparable with the *in vivo* conditions, where all healing parameters are functioning, long-term controlled and randomized success-and-failure clinical studies on patients are also necessary to assess *in vivo* responses when different sealers are extruded into the periradicular tissue. However, these types of studies can give dentists an opportunity to compare the relative toxicity of these filling materials.

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