

Effects of Salivary Acetylcholinesterase on the Cytotoxicity of Acrylic Reline Resins

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EFFECTS OF ACETYLCHOLINESTERASE ON THE CYTOTOXICITY OF ACRYLIC RELINE RESINS

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Introduction & Objectives

The use of autopolymerizing Acrylic Resin (AR) has recently gained popularity to replace dentures to the continuous modified underlying tissue. However, these materials have been associated with high levels of acidity and chemical irritation and direct reactions in oral epithelium to be caused by substances eluted from these materials, especially unreacted residual monomers (RM), which remained in the resin net after polymerization. These biomonomers are subject of degradation in the oral cavity, to which enzymatic activity of salivary esterase plays an important role, particularly the acetylcholinesterase (AChE).

The main objective of this study was to assess the effect of a salivary esterase on the cytotoxicity of two direct ARs, Resinex and UG Gel Hard, and one indirect AR, Probase Gel, using two cytotoxicity functional assays, through the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to methylthiazolol-diphenylmethane (MTT) and by the release of a soluble optically opaque, tartrazine-dependent dye (TZ), into the cell culture medium as the marker for membrane damage.

In order to get a clear insight of the role of the monomers on the cytotoxicity of these materials, it was also assessed the cellular viability after exposure to isolated methylacrylate (MMA), hexamethylenediamine (HMDA), methacryloyloxyethyl methacrylate (MMA) and the common hydrolysis by-product methacrylic acid (MA), using into account the IC50 (half maximal inhibitory concentration) of a substance. The IC50 of the ARs liquids was also studied.

Materials and Methods

Test Specimens: The specimens were prepared according to ISO recommendation for biological evaluation of biomaterials. Direct ARs were set at 37°C during the recommended polymerization time, in order to obtain the *in vitro* and oligomerization of the material. Polymerization of indirect AR was carried out in an heated porous device (boiler HydroLab, Calverton) for the recommended time, temperature and pressure.

Cell Viability: After UV irradiation, specimens of each material (n=6) were randomly divided into two groups: experimental, immersed in 3 mL of serum-free DMEM with 3.125 µL of AChE, and control, immersed only in 3 mL of serum-free DMEM. Specimens were incubated for 72 h at 37°C, under constant agitation to allow the soluble components to leach into the medium. Every 24 h, 3 µL/mL of AChE was added to experimental specimens. In order to measure the enzyme activity and DMEM was added to control specimens. Negative, positive and enzyme control (enzyme without cells) were also included. All specimens' eluates were then diluted in fresh experimental DMEM as follows: no dilution (100%), 1:4 dilution (75%) and 1:8 dilution (50%).

Cytotoxicity Assays: Cells were inoculated into 96-well culture plates at a density of 3.2x10³ cells/well and incubated at 37°C under a 5% CO₂ atmosphere. After 24 h, the supernatant was removed and cells were treated for a further 24 h period with 200 µL per well of serial dilutions of the eluates (n=6) per combination. After the 24 hours incubation, both MTT and TZ dye assays were conducted accordingly.

Concentrations and ARs liquids: At least seven concentrations of each liquid and compound were diluted in DMEM supplemented with ethanol at a final concentration of 0.5%, in order to obtain the IC50. These samples were measured only by the MTT assay. IC50 was determined using a non-linear regression of Dose-Response - Inhibition type (sigmoidal) to normalized response - variable model.

Statistical Analysis: Mann-Whitney tests were used to compare control and AChE groups. To compare materials, test compounds and dilutions was performed Kruskal-Wallis test, followed by post-hoc testing Tukey multiple comparison. A value of 0.05 was considered significant.

Results

1. The indirect AR Probase Gel eluates demonstrated no cytotoxic effect to human fibroblasts (Figure 1).
 2. Both direct ARs revealed cytotoxicity to human fibroblasts. Resinex specimens showed to be severely cytotoxic, and UG Gel Hard specimens moderately cytotoxic (Figure 1).
 3. Incubation with AChE did not change the non-cytotoxic effect of Probase Gel (Figure 1).
 4. Incubation with AChE caused a slight increase on cell viability of both direct ARs (Resinex and UG Gel Hard), without changing their cytotoxic potential (Figure 1).
 5. Both indirect ARs showed a dose-dependent effect on cytotoxicity for the Resinex and UG Gel Hard eluates (Figure 1).
 6. UChE activity didn't suffer changes between groups (Figure 2).
 7. The viability of the cells exposed to the pure compounds decreased in order: HMDA > MMA > MMA. MMA showed the cytotoxicity at the concentrations used (Figure 4).
 8. The direct ARs liquids and respective pure compounds exhibited similar behavior (Figure 1 and 4).

Conclusion

Acetylcholinesterase didn't change the cytotoxic potential of the reliner resins studied. Probase Gel revealed to be the less cytotoxic material. The cytotoxicity of the monomers alone can't explain the cytotoxicity of the materials studied.

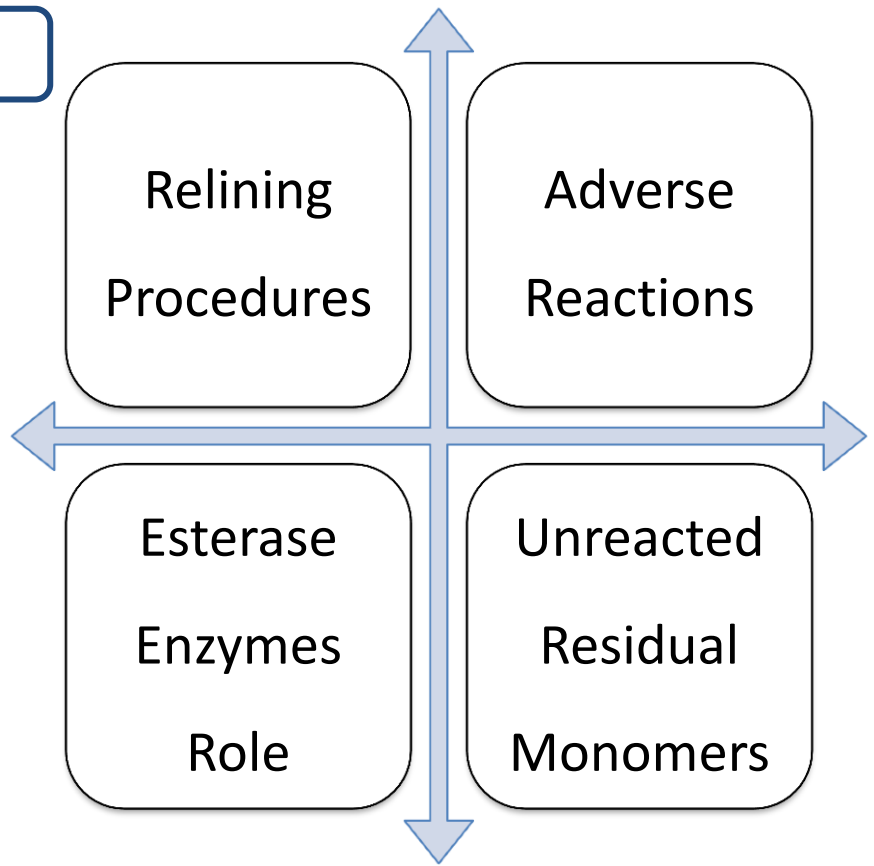
Financial Support

Financial support was provided by FCT (PTDC/SAU/114007/2009) and FCT (PTDC/SAU/114007/2009).

Abbreviations

AR: Acrylic Resin; AChE: Acetylcholinesterase; BMA: Butyl Methacrylate; DMEM: Dulbecco's Modified Eagle's Medium; HEMA: Hexamethylenediamine; IC50: Half Maximal Inhibitory Concentration; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MMA: Methyl Methacrylate; UG Gel Hard: UG Gel Hard; UChE: Urokinase-type plasminogen activator; TZ: Tartrazine.

State of the Art



Bohnenkamp DM. Traumatic stomatitis following an intraoral denture reliner: a clinical report. J Prosthet Dent 1996; 76:113-114; Huang FM, Tai KW, Hu CC, Chang YC. Cytotoxic effects of denture base materials on a permanent human oral epithelial cell line and on primary human oral fibroblasts in vitro. Int J Prosthodont 2001; 14:439-443; Bettencourt AF, Neves CB, Almeida MA, Pinheiro LM, Oliveira SA, Lopes LP, Castro MF. Biodegradation of acrylic based resins: A review. Dent Mater 2010; 26: e171-e180

Effects of Salivary Acetylcholinesterase on the Cytotoxicity of Acrylic Resine Resins

EFFECTS OF ACETYLCHOLINESTERASE ON THE CYTOTOXICITY OF ACRYLIC RELINE RESINS

M Mendes de Oliveira¹, LP Lopes¹, J Miranda², M Castro³, AF Bettecourt⁴, CB Neves⁵

Introduction & Objectives

The use of autopolymerizing Acrylic Resine Resins (ARR) has recently gained popularity to replace dentures to the continuous modified underlying tissue. However, these materials have been associated with high levels of acidity and chemical irritative and direct cytotoxic reactions in oral epithelium to be caused by substances eluted from these materials, especially unreacted residual monomers (RM), which remained in the resin net after polymerization. These biomonomers are subject of degradation in the oral cavity, to which acetylcholinesterase activity of salivary glands participate in the biotransformation (ACET).

The main objective of this study was to assess the effect of a salivary enzyme on the cytotoxicity of two direct ARR, Kooliner and Ufi Gel Hard, and one indirect ARR, Probase Cold, using two cytotoxicity functional assays, through the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to tetrahydrodiazepines (MTT) and by the release of a soluble optically opaque, tartrazine-dependent (DTZ), from the culture medium as the marker for membrane damage.

In order to get a clear insight of the role of the reduction on the cytotoxicity of these materials, it was also assessed the cellular viability after exposure to isolated acetylcholinesterase (AChE), hexachlorocyclopentadiene (HCCP), and the common byproducts by-product methacrylic acid (MA), using also the IC50 (Half maximal inhibitory concentration) of a substrate. The IC50 of the ARR liquids was also studied.

Materials and Methods

Test Specimens: The specimens were prepared according to ISO recommendation for biological evaluation of biomaterials. Direct ARR were set at 37°C during the conventional polymerization time, in order to minimize the in vitro and in vivo degradation of the material. Polymerization of indirect ARR was carried out in a heated pressure device (vacuum chamber, 1 atmosphere) for the recommended duration, temperature and pressure.

Test Objects: After identification, specimens of each material (n=6) were randomly divided into two groups: experimental, immersed in 1 mL of serum-free DMEM with 1.1 mg/L of AChE, and control, immersed only in 1 mL of serum-free DMEM. Specimens were incubated for 72 h at 37°C, under constant agitation to allow the soluble components to leach into the medium. Every 24 h, 5 µL/mL of AChE was added to experimental specimens, in order to maintain the enzyme activity and DMEM was added to control specimens. Negative, positive and enzyme control (achar) specimens were also included. All specimens eluates were then diluted in fresh experimental DMEM as follows: no dilution (100%), 1:4 dilution (75%) and 1:2 dilution (50%).

Cytotoxicity Assays: Cells were inoculated into 96-well culture plates at a density of 3.2x10³ cells/well and incubated at 37°C under a 5% CO₂ atmosphere. After 24 h, the supernatant was removed and cells were treated for a further 24 h period with 200 µL per well of serial dilutions of the eluates (n=6) per concentration. After the 24 hours incubation, both MTT and DTZ tests were carried out.

Compound Solutions and ARR liquids: At least seven concentrations of each liquid and compound were diluted in DMEM supplemented with ethanol at a final concentration of 0.25%, in order to obtain the IC50. Three samples were measured only by the MTT assay. IC50 was determined using a non-linear regression of Dose-Response - Inhibition type (log-log) using the non-linear least-squares method - variable slope.

Statistical Analysis: Mann-Whitney tests were used to compare control and AChE groups. To compare materials, test compounds and dilutions was performed Kruskal-Wallis test, followed by post-hoc testing Tukey multiple comparisons. A p-value < 0.05 was considered significant.

Results

MTT assay of eluates:

MTT assay of HEMA:

MTT assay of BMA:

MTT assay of MA:

Ufi Gel Hard vs HEMA:

Kooliner vs BMA:

Probase Cold vs MA:

Conclusion

Acetylcholinesterase didn't change the cytotoxic potential of the reline resins studied. Probase Cold revealed to be the less cytotoxic material. The cytotoxicity of the monomers alone can't explain the cytotoxicity of the materials studied.

Financial Support

Bibliography

Materials & Methods

Kooliner (K)

GC America Inc

Ufi Gel Hard (U)

VocoGmbH

Compounds Solutions

liquids

Specimens

Statistical Analysis

Probase Cold (P)

Ivoclar Vivadent AG

Eluates

Cytotoxicity Assays

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Introduction & Objectives

The use of autopolymerizing acrylic resin (AR) has recently gained popularity to replace dentures in the continuous resurfaced underlying tissue. However, these materials have been associated with high levels of acidity in vitro and chemical irritation and direct cytotoxic in vivo, followed by the release of substances eluted from these materials, especially unreacted residual monomers (RM), which remained in the resin not after polymerization. These biomaterials are subject of degradation in the oral cavity, in which normally activity of salivary acetylcholinesterase (AChE) is present.

The main objective of this study was to assess the effect of a salivary enzyme on the cytotoxicity of two direct AR, Kooliner and Ufi Gel Hard, and one indirect AR, Probace Cold, using two common functional assays, through the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3,4-diphenylthio)carbazole by mitochondrial dehydrogenase (MTT) and by the release of a soluble cytochrome enzyme, lactate dehydrogenase (LDH), into the cell culture medium as the marker for membrane damage.

In order to get a clear insight of the role of the presence of these materials, it was also assessed the cellular viability after exposure to isolated acetylcholinesterase (AChE), hexacarbonylchloride (HgCl₂) and the common hydrolysis by-product methacrylic Acid (MA), using into account the IC50 (half maximal inhibitory concentration) of a substrate. The IC50 of the ARR liquids was also studied.

Materials and Methods

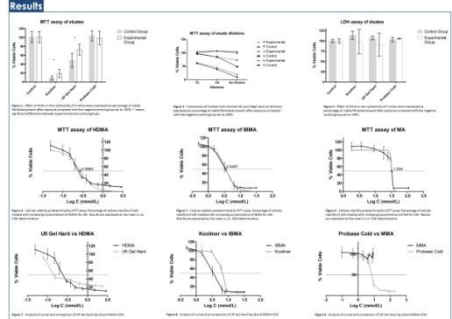
Cell Specimens: The specimens were prepared according to ISO recommendation for biological evaluation of biomaterials. Direct ARR were set at 37°C during the conventional polymerization. The in vitro and in vivo polymerization of the material. Polymerization of indirect ARR was carried out in an infrared power device (Infrared Heater, Calsciences) for 10 minutes at 100°C.

MTT Assay: After UV irradiation, specimens of each material (1-cm were randomly divided into two groups: experimental, immersed in 3 mL of serum-free DMEM with 3.125 µg/mL of AChE, and control, immersed only in 3 mL of serum-free DMEM. Specimens were incubated for 72 h at 37°C, under constant agitation to allow the soluble components to leach into the medium. Every 24 h, 3 µL/mL of AChE was added to experimental specimens. In order to measure the enzyme activity and AChE was added to control specimens. Negative, positive and enzyme control (enzyme) specimens were also included. All specimens' eluates were then diluted in fresh experimental DMEM as follows: no dilution (100%), 1:4 dilution (75%) and 1:8 dilution (50%).

Cytotoxicity Assays: Cells were inoculated into 96-well culture plates at a density of 3.2x10³ cells/well and incubated at 37°C under a 5% CO₂ atmosphere. After 24 h, the supernatant was removed and cells were then treated for a further 24 h period with 200 µL per well of serial dilutions of the eluates (50% per combination). After the 24 hours incubation, both MTT and LDH assays were conducted accordingly.

Compound solutions and ARR leachates: At least seven concentrations of each liquid and compound were diluted in DMEM supplemented with ethanol at a final concentration of 0.5%, in order to obtain the IC50. These samples were measured only by the MTT assay. IC50 was determined using a non-linear regression of Dose-Response - Inhibition type (sigmoidal) to normalized response - variable model.

Statistical analysis: Mann-Whitney tests were used to compare control and AChE groups. To compare materials, two compounds and dilutions was performed Kruskal-Wallis test, followed by post-hoc testing Tukey multiple comparison. A value of 0.05 was considered significant.



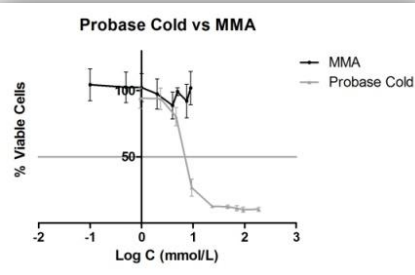
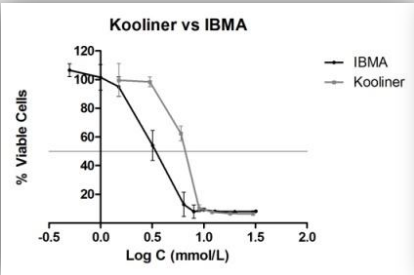
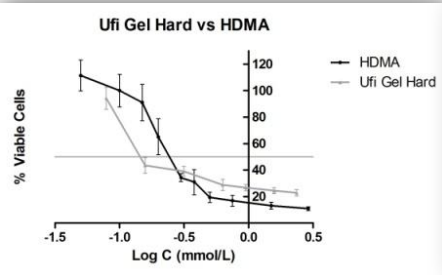
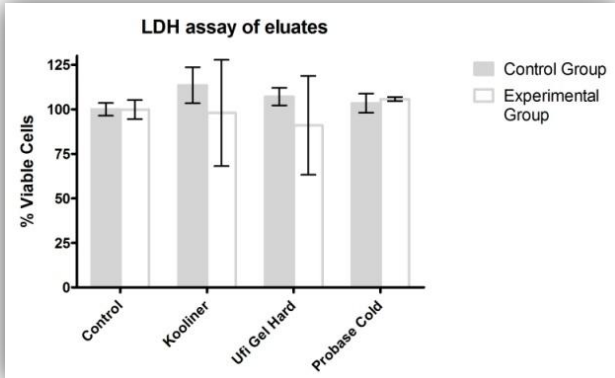
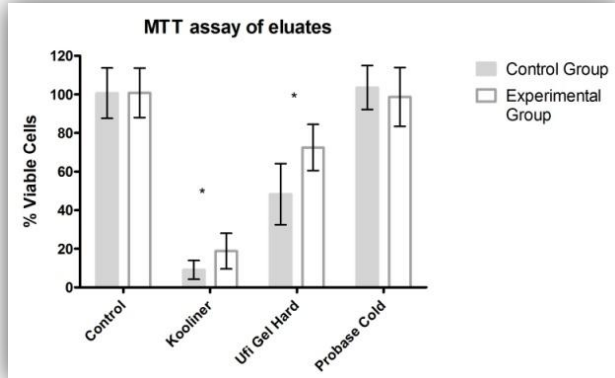
Conclusion

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Financial Support

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Results



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Introduction & Objectives

The use of autopolymerizing Acrylic Resine Resins (ARR) has recently gained popularity to replace dentures to the continuous reworked underlying tissue¹. However, these materials have been associated with high levels of acidity and chemical irritation and direct cytotoxicity in oral epithelium to be caused by substances eluted from these materials, especially unreacted residual monomers (RM), which remained in the resin net after polymerization². These biomaterials are subject of degradation in the oral cavity, in which enzymatic activity of salivary enzymes is important, in particular the acetylcholinesterase (AChE)³.

The main objective of this study was to assess the effect of a salivary enzyme on the cytotoxicity of two direct ARR, Resinac and UL Gel Hard, and one indirect ARR, Probace Cold, using two cytotoxicity functional assays, through the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3,4-diphenylthio)carbazole by mitochondrial dehydrogenase (MTT) and by the release of a soluble optically opaque, lactate dehydrogenase (LDH), into the cell culture medium as the marker for membrane damage.

In order to get a clear insight of the role of the monomers on the cytotoxicity of these materials, it was also assessed the cellular viability after exposure to isolated methacrylates (BMA, hexamethacrylate (HMA) and methyl methacrylate (MMA)) and the common backbone by-product methacrylate acid (MA), using also acetate the IC50 (half maximal inhibitory concentration) of a substance. The IC50 of the ARR liquids was also studied.

Materials and Methods

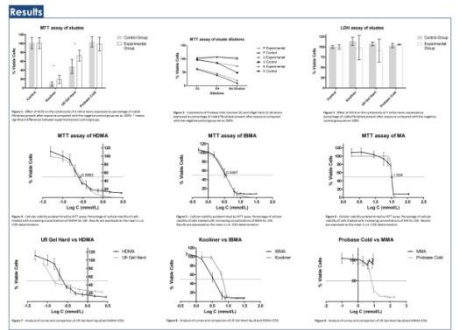
Test Specimens: The specimens were prepared accordingly to ISO recommendation for biological evaluation of biomaterials⁴. Direct ARR were set at 37°C during the conventional polymerization time, in order to simulate the *in vivo* and polymerization of the material. Polymerization of indirect ARR was carried out in an heated pressure device (Isolar, Haverhill, USA) according to the recommendations, temperature and pressure.

Test Objects: After 24 h incubation, specimens of each material (n=6) were randomly divided into two groups: experimental, immersed in 3 mL of serum-free DMEM with 1.1 µM of AChE, and control, immersed only in 3 mL of serum-free DMEM. Specimens were incubated for 72 h at 37°C, under constant agitation to allow the soluble components to leach into the medium. Every 24 h, 3 µL/mL of AChE was added to experimental specimens. In order to maximize the enzyme activity and BMA and HMA was added to control specimens. Negative, positive and enzyme control (enzyme without cells) were also included. All specimens' eluates were then diluted in fresh supplemented DMEM as follows: control (100%), 1:4 dilution (75%) and 1:2 dilution (50%).

Cell Viability Assays: Cells were inoculated into 96-well culture plates at a density of 3.2x10³ cells/well and incubated at 37°C under a 5% CO₂ atmosphere. After 24 h, the supernatant was removed and cells were then treated for a further 24 h period with 200 µL per well of serial dilutions of the eluates (n=6) per combination. After the 24 hours incubation, both MTT and LDH assays were conducted accordingly.

Compound solutions and ARR liquids: At least seven concentrations of each liquid and compound were diluted in DMEM supplemented with ethanol at a final concentration of 0.5%, in order to obtain the IC50. These samples were measured only by the MTT assay. IC50 was determined using a non-linear regression of Dose-Response - Inhibition type (sigmoidal) to normalized response - variable model.

Statistical analysis: Mann-Whitney tests were used to compare control and AChE groups. To compare materials, two compounds and dilutions was performed Kruskal-Wallis test, followed by post-hoc testing Tukey multiple comparisons. A p-value < 0.05 were considered significant.



- The indirect ARR Probace Cold eluates demonstrated no cytotoxic effect to human fibroblasts (Figure 1).
- Both direct ARR revealed cytotoxicity to human fibroblasts. Resinac specimens showed to be severely cytotoxic, and UL Gel Hard specimens moderately cytotoxic (Figure 1).
- Inclusion with AChE did not change the non-cytotoxic effect of Probace Cold (Figure 1).
- Incubation with AChE caused a slight increase on cell viability of both direct ARR (Resinac and UL Gel Hard), without changing their cytotoxic potential (Figure 1).
- Both indirect and direct ARR showed no cytotoxicity to the fibroblast and UL Gel Hard eluates (Figure 1).
- LDH activity didn't suffer changes between groups (Figure 2).
- The viability of the cells exposed to the pure compounds decreased in order: HDMA > IBMA > MA. MMA showed no cytotoxicity at the concentrations used (Figure 4).
- The direct ARR liquids and respective pure compounds exhibited similar behavior (Figure 3 and 4).

Conclusion

Acetylcholinesterase didn't change the cytotoxic potential of the reliner resins studied. Probace Cold revealed to be the less cytotoxic material. The cytotoxicity of the monomers alone can't explain the cytotoxicity of the materials studied.

Financial Support: Fundação para a Ciência e Tecnologia (Portugal): (PEst-OE/SAU/UI4062/2011; PEst-OE/SAU/UI4013/2011; Ciência 2008 for J. P. M. and EXCL/CTMNaN/0166/2012).

Conclusions

Acetylcholinesterase didn't change the cytotoxic potential of the reliner resins studied.

Probace Cold was the less cytotoxic material.

HDMA > IBMA > MA

Financial Support: Fundação para a Ciência e Tecnologia (Portugal): (PEst-OE/SAU/UI4062/2011; PEst-OE/SAU/UI4013/2011; Ciência 2008 for J. P. M. and EXCL/CTMNaN/0166/2012).