Original article

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APPLICATION OF DIRECT CONTACT TEST IN EVALUATION OF CYTOTOXICITY OF ACRYLIC DENTURE BASE RESINS

Milena Kostić[1], Stevo Najman[2], Jelena Najdanović[2], Nebojša Krunić[3] and Ivan Kostić[2]

The use of acrylic denture base resins is widely spread in dental practice. They belong to the group of biomaterials due to their role of morphological and functional substituent in the mouth. However, clinical practice has shown that some toxic ingredients of these materials may lead to adverse local and even systemic changes.

The aim of the study was to evaluate cytotoxic effect of various denture base resins on cell culture using direct contact test.

The effect of four different acrylic materials on HeLa cell structure was evaluated. Upon light microscopy analysis, MTT test was performed without previous removal of material samples.

The obtained values of MTT indicate that cell proliferation is dependant on the type of acrylic denture base resins. Cold polymerization denture base resins showed mild inhibitory effect on the cell culture growth. The signs of toxicity were not observed in heat polymerization denture base resins. *Acta Medica Medianae 2012; 51(1):66-72.*

Key words: *acrylic denture base resins, direct contact test, HeLa cells*

Clinic of Dentistry Niš, Department of Prosthodontics, Niš, Serbia[1]
University of Niš, Faculty of Medicine, Institute of Biology and Human Genetics, Niš, Serbia[2]
Clinic of Dentistry Niš, Department of Prosthodontics, Serbia, Niš[3]

Contact: Milena Kostić
Clinic of Dentistry Niš, Department of Prosthodontics
Institute of Biology and Human Genetics,
Blvd dr Zoran Đinđić 52, 18000 Niš, Serbia
E-mail: kosticmilena@sbb.rs

Introduction

Acrylic denture base resins have been used for the past eighty years in dental practice and are still indispensable in their area of indication (1). As building materials they are used for construction of plate part of mobile dentures, false teeth, temporary crowns and bridges, mobile orthodontic appliances, obturators and maxillofacial prostheses, as well as for relining and repair (2). They are referred to as biomaterials due to their role of morphological and functional substituent in the mouth (3, 4). However, clinical practice has shown that some toxic ingredients of acrylates may lead to adverse local and even systemic changes (5-8). The degree of tissue sensitivity to acrylate increases with the percentage increase of potentially toxic substances in the material (9, 10). These substances have the ability to leak from denture and diffuse in saliva by means of which they affect oral mucosa (11-14). The amount of potentially toxic substances in acrylic material is different and depends on the type and time of polymerization (11, 15-17).

Evaluation of biological properties in vitro provides adequate comparison of various acrylates commercially available, and subsequent easier selection of materials and polymerization procedure in everyday work with patients. The contact between cell culture and tested material is achieved in direct and indirect way by means of extracts (18). In case of direct contact, cytotoxicity is measured by the rate of cell death in the function of exposure time and distance from the sample which is in solid physical condition (19). The most frequent complications in vitro tests with direct contacts include bacterial contamination of culture and mechanical damage of cells by direct contact with the material (19,20).

The aim of the study was to evaluate cytotoxic effect of various denture base resins on cell culture using direct contact test..

Material and methods

Tested material

The tested material involved three hard and three soft denture base resins used in dental practice for construction and readaptation of mobile dentures. Cold, heat and light polymerization denture base resins were used in the study (Table 1).

Six samples of each tested acrylic material were made, the shape and size of which matched the half of the separate field bottom on the cultivation plate with 48 wells (semicircle with 1cm diameter). The material for cultivation was paste-like, so that the final bond was done within individual wells. After having been polymerized samples of heat polymerization acrylates were inserted in the plate wells for cell cultivation (Figure 1). Models of the samples were originally made in pink dental wax (Vomogal-S, Galenika, Serbia). Samples for evaluation were made immediately before each experiment to avoid possible changes resulting from material aging.

Table 1. Tested denture base acrylic resins

Tested material	Manufacturer	Acrylic type	Content	
			powder	liquid
Bosforth Trusoft	HG Bosworth Company USA	soft cold polymerized acrylate	poly(ethyl methacrylate)	ethyl alcohol, butyl benzyl phthalate
Lang Flexacryl	Lang Dental MFG.Co. USA	soft cold polymerized acrylate	poly(ethyl methacrylate)	n-buthyl methacrylate
Lang Immediate	Lang Dental MFG.Co. USA	soft cold polymerized acrylate	poly(ethyl methacrylate)	methyl methacrylate
Lucitone 199	Dentsply International Inc. USA	light polymerized acrylate	poly(methyl methacrylate)	methyl methacrylate
Triplex Cold	Ivoclar Vivadent, Lichtenstein	hard cold polymerized acrylate	poly(methyl methacrylate)	methyl methacrylate, ethylene glycol dimethacrylate
Triplex Hot	Ivoclar Vivadent, Lichtenstein	heat polymerized acrylate	poly(methyl methacrylate)	methyl methacrylate, ethylene glycol dimethacrylate

Figure 1. Polymerization of the heat polymerized acrylic samples in standard metal flask (Changsha Zhongbang Medical Instruments Co., China).

HeLa cell culture

We used HeLa cell lines preserved in feeding medium DMEM (Dulbecco's Modified Eagle's Minimal Essential Medium, PAA Laboratories GmbH) enriched with the addition of l-glutamine, penicillin-streptomycine (100 IU / ml) and 10% fetal bovine serum (FBS). Each procedure with cells was done in vertical sterile chamber (Bioair Instruments, Italy). The cell culture was maintained in the incubation (Binder, Germany), in the atmosphere saturated with water vapor, with 5% CO2, at the temperature of 37 º C.

Experimental design

Cultivation plate with 48 wells containing samples of tested acrylic material was sterilized in a vertical chamber with the one-day action of UV rays (21).

Planting of 104 cells in 100 µl of nutritive medium in each well was followed by a one-day incubation of cells and materials in the atmosphere saturated with water vapor, with 5% CO2 at 37°C. The control group consisted of cells grown without the present material. After microscopic analysis the MTT test was done without removing the samples of materials.

Light microscopy

Viability, density and epithelial organization of HeLa cells were observed under the invert microscope (Observer Z1, Zeiss, Germany).

Cell density was determined by their counting in three vertically placed quadrants of visual field for each place of the tested sample and the control group. Cells with altered phenotype separated from the base were counted as dead.

Since the tested material occupied a quarter of each observed quadrant in the visual field, the density of cells in the control group was presented as 75% of its total cell growth.

MTT test

The enzyme succinate dehydrogenase that is an integral part of mytochondrial respiratory chain is active in viable cells. This enzyme reduces yellow tetrazolium salt - MTT (3 - (4,5-di metiltiazoil-2) - 2,5-diphenitetrasolium bromide) to formasan, a blue colored compound that is deposited in the cells in the form of crystals. Isopropanol damages cell membrane and formasan dissolves. There is a direct reciprocity between the number of viable cells and the intensity of blue colour (Figure 2).

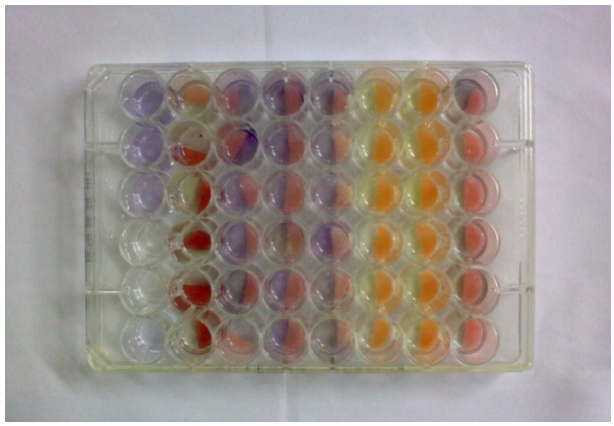


Figure 2. MTT test showed a direct proportionality between the number of living cells and the intensity of blue colour.

The medium in which cells were incubated was drawn at the end of the incubation and the cells were washed with 100 µl PBS (phosphate buffered salt) and 20 µl of MTT was added. After 4 h- incubation at 37 º C, the formasan crystals were dissolved with 100 µl of isopropanol. Spectro-photometric measurement of the intensity of MTT reduction was carried out at optical density of 540 nm on the multi-channel photometer (Multiskan Ascent No354, Thermo Labsystems, Finland).

Interpretation of results

Quantitative changes in cell proliferation are presented descriptively: non-cytotoxic (> 90%), slightly cytotoxic (60-90%), moderately cytotoxic (30-59%), severely cytotoxic (<30%) viability and growth of cells compared to the control group (22). Regarding the one-day incubation, the results are presented as percentage of cell viability and cell culture proliferation of tested groups compared to controls. Since HeLa cells are epithelial, adherent cells, the reduction of adherent phenotype was considered as the sign of toxic effect.

For the values obtained by MTT test statistical analysis of cytotoxicity of the tested materials compared with the control testing and mutual testing of samples was performed. We used ANOVA ($p < 0.05$) and Post Hoc Analysis.

Cell viability and proliferation of untreated control groups are presented as 100% cell growth.

Results

Figures 3-6 show cell viability, proliferation and epithelial organization in direct contact with the samples of tested materials. It may be noted that cells change shape when close to material.

Round cells separated from the base are regarded dead.

The greater the distance from the sample material the greater the density of HeLa cells that are organized in clusters characteristic for epithelial tissue.

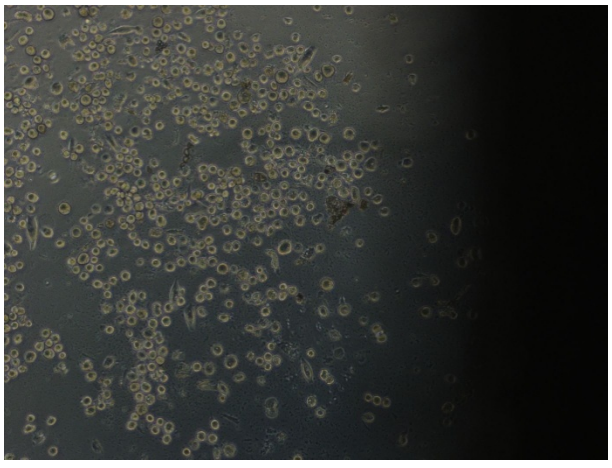


Figure 3. The growth and organization of epithelial HeLa cells in the presence of Bosworth Trusoft samples (magnification ×20).

Graph 1. shows the percentage of live HeLa cells cultured in contact with the samples of tested acrylic materials compared to controls. The control group is presented as 75% of cell growth.

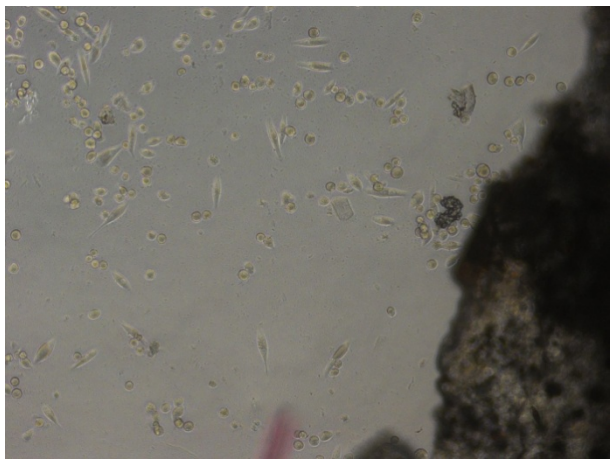


Figure 4. The growth and organization of epithelial HeLa cells in the presence of Lucitone 199 samples (magnification ×20)

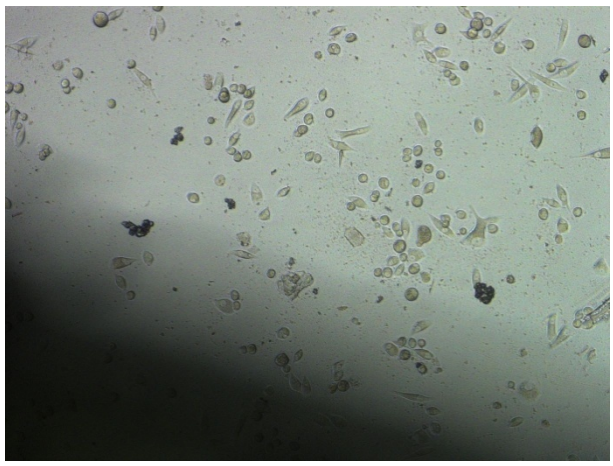


Figure 5. The growth and organization of epithelial HeLa cells in the presence of Triplex Cold samples (magnification ×20).

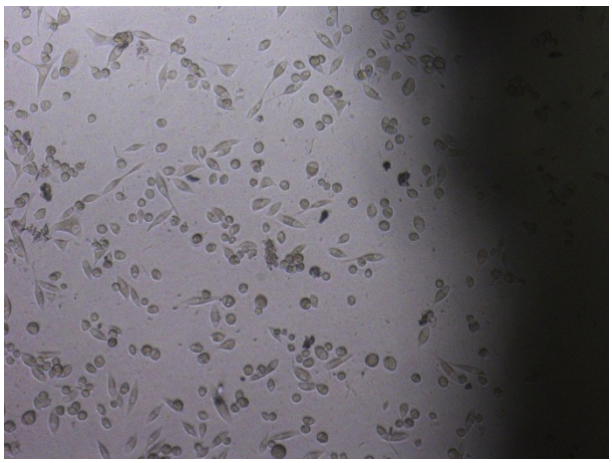
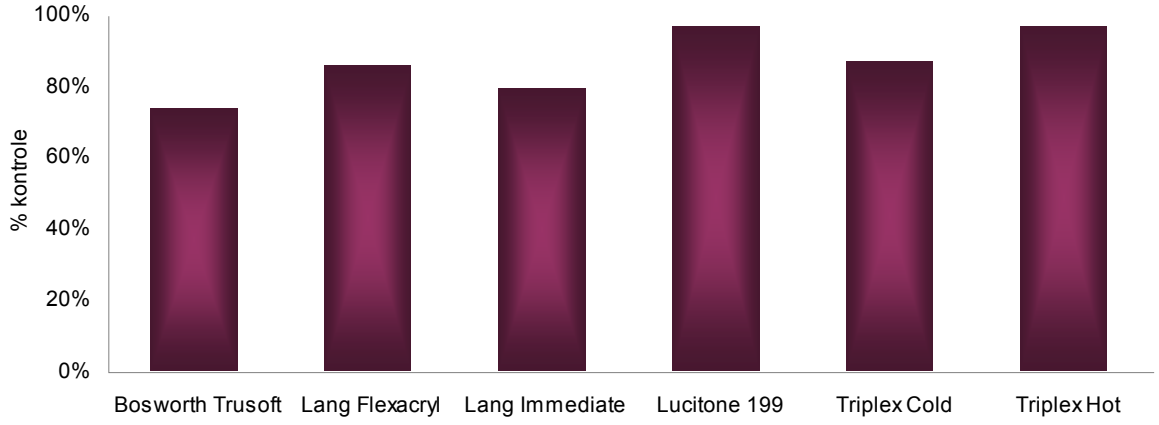
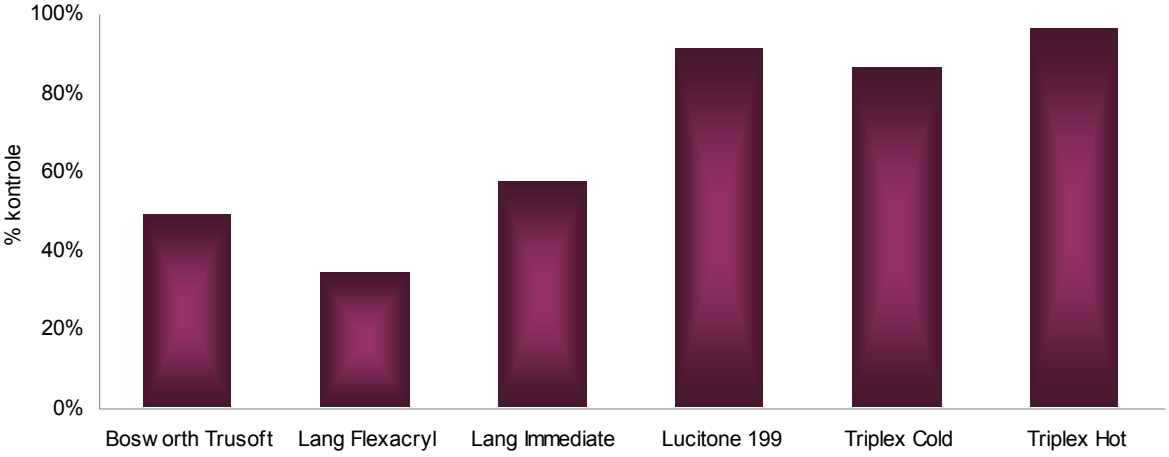


Figure 6. The growth and organization of epithelial HeLa cells in the presence of Triplex Hot samples (magnification ×20)



Graph 1. The percentage of viable cells compared to control, after one day direct contact with the acrylic materials.



Graph 2. The percentage of epithelial organization compared to control, after one day direct contact with the acrylic materials.

Tabela 2. Procenat intenziteta redukcije MTT-a ispitivanih akrilatnih materijala u odnosu na kontrolu

Ispitivani materijal	N	X	±	SD	Cv	SE	95	%	CI	Min	Max
Bosworth Trusoft	6	78,71	±	8,72	11,08	3,56	69,56	–	87,85	90,71	11,08
Lang Flexacryl	6	98,42	±	22,72	23,08	9,27	74,58	–	122,26	118,58	23,08
Lang Immediate	6	79,94	±	15,05	18,82	6,14	64,15	–	95,73	105,24	18,82
Lucitone 199	6	96,35	±	7,04	7,31	2,88	88,95	–	103,74	106,13	7,31
Triplex Cold	6	96,40	±	13,80	14,31	5,63	81,91		110,88	120,95	14,31
Triplex Hot	6	* 99,70	±	9,51	9,54	3,88	89,73		109,68	110,57	9,54

* - p<0,05 vs Bosworth Trusoft

Figure 2 shows the degree of epithelial organization of HeLa cell cultures in direct contact with samples of acrylic material compared to control group. The control group is presented as 75% of the total epithelial organization of epithelial HeLa cells

The obtained values of MTT test indicate percentage dependance of cell viability and proliferation in relation to the type of acrylic material (ANOVA, p<0,05). Using Tamhan test and Post hoc analysis it was determined that there was the greatest intensity of MTT reduction for tested samples of Triplex Hot and statistical significance in relation to Bosworth Trusoft samples.

The lowest intensity of MTT was found in Bosworth Trusoft samples. Tamhan test showed that this difference was very close to the threshold of statistical significance (p=0,0505) compared to the tested Lucitone 199.

Table 2. shows the intensity of reduction of MTT in relation to the type of tested acrylic material.

Discussion

The analyses of cell culture are of great importance for understanding biological action of the material. However, they are prone to certain limitations referring primarily to impossibility of

simulation of in vivo situation and difficulty in withdrawing credible conclusions from the obtained results. Therefore, they represent only initial or screening method of evaluation of biocompatibility of both new materials and those already in clinical use.

Potentially toxic effect of acrylates has been explained by direct contact between tested materials and cells in culture. HeLa cells can be regarded as analogues of the epithelial cells of oral mucosa, what is, apart from possibility of their successful cultivation in laboratory conditions and easy multiplication, the reason why they were chosen for this study. The cells were planted directly on the samples, which proved to be the most efficient method of evaluating materials in vitro. Cellular reaction, in this case, reflects not only cytotoxic tissue response but also depends on the features of material surface (19).

The cells close to material changed their shape. Round cells separated from the base were regarded dead. The greater the distance from the sample material the greater the density of HeLa cells that are organized in clusters characteristic for epithelial tissue.

On the basis of the obtained data, it is obvious that there is mild toxic effect of soft acrylates and Triplex Cold on HeLa cell culture, that is, their clinical application immediately leads to reduction of proliferation of epithelial cells (73-87%). Conversely, the direct contact test showed non-toxic effect of Lucitone and Triplex Hot 199 (97%).

Regarding the fact that epithelial clustering of cells is characteristic of HeLa culture, any deviation from the normal phenotype organization could be considered to have negative effect on applied material. The lowest percentage of clustering of HeLa cells was observed in direct contact of culture with soft acrylates (35-60%). The presence of cold polymerized Triplex Cold (85%) showed a slightly lower level of cell organization in relation to light-polymerized Lucitone 199 (92%). The greatest degree of epithelial organization was observed in Triplex Hot (96%), which indicates its negligible cytotoxicity.

The growth of cells in culture is presented by the intensity of MTT reduction as well. The obtained MTT values indicate that cell proliferation is dependant on the type of acrylic denture base resins. The lowest values are present in Trusoft Bosworth, and the highest values are present in Triplex Hot. Excellent biological characteristics of Triplex Hot may be attributed to its flat surface structure, and minimal porosity (16). The results obtained are in accordance with the results of in vitro examination of potential acrylic toxicity on culture using the indirect contact test (22-29). On the other hand, in vitro examinations by Huang et al. and Melili et al. confirmed greater toxicity of the heat polymerized acrylic denture base resins in comparison to light polymerized acrylic denture base resins (30, 31). Cold polymerized Triplex Cold had higher inhibitory potential as compared to heat and light polymerized resins, which is in accordance with the results of other authors as well (28,29, 32,33). The study results showed greater inhibitory effect of soft resins compared to other studied groups, which correlates with the findings of Okita et al. (34). Increased cytotoxicity of cold polymerized denture based resins can be explained by their weaker and more porous structure (35,36).

Conclusion

Acrylic denture base resins may be considered to have biocompatible features, which justifies their everyday use in clinical practice. Cold polymerized denture-based resins showed more toxic effect on cell structure in comparison to heat and light polymerized acrylic denture-based resins.

References

1. Krunić N, Kostić M, Anđelković M. Akrilati-još uvek nezamenjivi materijali u stomatološkoj protetici. Acta Stomatol Naissi. 2007 ; 23(56): 747-52.
2. Strang R, Whitters CJ, Brown D, Clarke RL, Curtis RV, Hatton PV, et al. Dental materials: 1996 literature review. Part 2. J Dent. 1998 ; 26(4): 273-91. [CrossRef] [PubMed]
3. Frazer RQ, Byron RT, Osborne PB, West KP. PMMA: an essential material in medicine and dentistry. J Long Term Eff Med Implants. 2005 ; 15(6): 629-39. [PubMed]
4. Veličković S, Stamenković D. Polimerni materijali u stomatologiji. In: Stamenković D i sar, editors. Gradivni stomatološki materijali (dostignuća i perspektive). Beograd: Stomatološki fakultet; 2007. Serbian.
5. Freitas JB, Gomez RS, De Abreu MH, Ferreira E Ferreira E. Relationship between the use of full dentures and mucosal alterations among elderly Brazilians. J Oral Rehabil. 2008 ; 35(5): 370-4. [CrossRef] [PubMed]
6. LeSueur BW, Yiannias JA. Contact stomatitis. Dermatol Clin. 2003 ; 21(1): 105-14. [CrossRef] [PubMed]
7. Sadoh DR, Sharief MK, Howard RS. Occupational exposure to methyl methacrylate monomer induces generalised neuropathy in a dental technician. Br Dent J. 1999 ; 186(8): 380-1. [CrossRef] [PubMed]
8. Davis CC, Squier CA, Lilly GE. Irritant contact stomatitis: a review of the condition. J Periodontol. 1998 ; 69(6): 620-31. [CrossRef] [PubMed]
9. Phoenix RD, Mansueto MA, Ackerman NA, Jones RE. Evaluation of mechanical and thermal properties of commonly used denture base resins. J Prosthodont. 2004 ; 13(1): 17-27. [CrossRef] [PubMed]
10. Wataha JC, Lockwood PE, Bouillaguet S, Noda M. In vitro biological response to core and flowable dental restorative materials. Dent Mater. 2003 ; 19(1): 25-31. [CrossRef] [PubMed]
11. Kostić M, Krunić N, Nikolić L, Nikolić V, Najman S, Kocić J. [Residual monomer content determination in some acrylic denture base materials and

possibilities of its reduction]. Vojnosanit Pregl. 2009 ; 66(3): 223-7. Serbian. [PubMed]
12. Koda T, Tsuchiya H, Hoshino Y, Takagi N, Kawano J. High-performance liquid chromatographic estimation of eluates from denture base polymers. J Dent. 1989 ; 17: 84-9. [CrossRef] [PubMed]
13. Lamb DJ, Ellis B, Priestley D. Loss into water of residual monomer from autopolymerizing dental acrylic resin. Biomaterials. 1982 ; 3: 155-9. [CrossRef] [PubMed]
14. Vallittu PK, Miettinen V, Alakuijala P. Residual monomer content and its release into water from denture base materials. Dent Mater. 1995 ; 11(6): 338-42. [CrossRef] [PubMed]
15. Krunić N, Nikolić Lj, Kostić M, Najman S, Nikolić V, Najdanović J. In vitro examination of oral tissue conditioners potential toxicity. Chem Ind. 2011 ; 65(6): 697-706.
16. Kostić M, Krunić N, Nikolić Lj, Nikolić V, Najman S, Kostić I, Rajković J, Manić M, Petković D. Influence of residual monomer reduction on acrylic denture base resins quality Chem Ind. 2011 ; 65(2): 171-7.
17. Bartoloni JA, Murchison DF, Wofford DT, Sarkar NK. Degree of conversion in denture base materials for varied polymerization techniques. J Oral Rehabil. 2000 ; 27(6): 488-93. [CrossRef] [PubMed]
18. Dee KC, Puleo DA, Bizios R. Wound healing. In: An introduction to tissue-biomaterial interactions. Hoboken, New Jersey: John Wiley & Sons Inc.; 2002. p.165-214. [CrossRef]
19. Polyzois GL. In vitro evaluation of dental materials. Clin Mater. 1994 ; 16(1): 21-60. [CrossRef] [PubMed]
20. Schmalz G. Use of cell cultures for toxicity testing of dental materials--advantages and limitations. J Dent. 1994 ; 22 Suppl 2: S6-11. [CrossRef] [PubMed]
21. Moharamzadeh K, Brook IM, Van Noort R. Biocompatibility of Resin-based Dental Materials. Materials. 2009 ; 2(2): 514-48. [CrossRef]
22. Nakamura M, Kawahara H. Long-term biocompati bility test of denture base resins in vitro. J Prosthet Dent. 1984 ; 52(5): 694-9. [CrossRef] [PubMed]
23. Dahl JE, Frangou-Polyzois MJ, Polyzois GL. In vitro biocompatibility of denture relining materials. Gerodontology. 2006 ; 23(1): 17-22. [CrossRef] [PubMed]
24. Jorge JH, Giampaolo ET, Machado AL, Pavarina AC, Carlos IZ. Biocompatibility of denture base acrylic resins evaluated in culture of L929 cells, Effect of polymerisation cycle and post-polymerisation treatments. Gerodontology. 2007 ; 24(1): 52-7. [CrossRef] [PubMed]
25. Campanha NH, Pavarina AC, Giampaolo ET, Machado AL, Carlos IZ, Vergani CE. Cytotoxicity of hard chairside reline resins: effect of microwave irradiation and water bath postpolymerization treatments. Int J Prosthodont. 2006 ; 19(2): 195-201. [PubMed]
26. Jorge JH, Giampaolo ET, Vergani CE, Machado AL, Pavarina AC, Carlos IZ. Cytotoxicity of denture base resins: effect of water bath and microwave post polymerization heat treatments. Int J Prosthodont. 2004 ; 17(3): 340-4. [CrossRef] [PubMed]
27. Vallittu PK, Ekstrand K. In vitro cytotoxicity of fibre-polymethyl methacrylate composite used in dentures. J Oral Rehabil. 1999 ; 26(8): 666-71. [CrossRef] [PubMed]
28. Lefebvre CA, Knoernschild KL, Schuster GS. Cytotoxicity of eluates from light-polymerized denture base resins. J Prosthet Dent. 1994 ; 72(6): 644-50. [CrossRef] [PubMed]
29. Kostić M, Najman S, Kocić J, Krunić N, Ajduković Z, Petrović D, Anđelković M. Efekat ekstrakata akrilata za bazu pločaste zubne proteze na rasta HeLa ćelija in vitro. Hemijska industrija. 2008 ; 62(3): 217-22.
30. 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(5): 439-43. [PubMed]
31. Melilli D, Curro G, Perna AM, Cassaro A. Cytotoxicity of four types of resins used for removable denture bases: in vitro comparative analysis. Minerva Stomatologica. 2009 ; 58(9): 425-34. [PubMed]
32. Gough JE, Downes S. Osteoblast cell death on methacrylate polymers involves apoptosis. J Biomed Mater Res. 2001 ; 57(4): 497-505. [CrossRef] [PubMed]
33. Cimpan MR, Matre R, Cressey LI, Tysnes B, Lie SA, Gjertsen BT, Skaug N. The effect of heat- and auto-polymerized denture base polymers on clono genicity, apoptosis, and necrosis in fibroblasts: denture base polymers induce apoptosis and necrosis. Acta Odontol Scand. 2000 ; 58(5): 217-28. [CrossRef] [PubMed]
34. Okita N. In vitro cytotoxicity of tissue conditioners. J Prosthet Dent. 1991 ; 66: 656-9. [CrossRef] [PubMed]
35. Bayraktar G, Guvener B, Bural C, Uresin Y. Influence of polymerization method, curing process, and length of time of storage in water on the residual methyl methacrylate content in dental acrylic resins. J Biomed Mater Res B Appl Biomater. 2006 ; 76(2): 340-5. [CrossRef] [PubMed]
36. Lung CY, Darvell BW. Methyl methacrylate monomer-polymer equilibrium in solid polymer. Dent Mater. 2007 ; 23(1): 88-94. [CrossRef] [PubMed]

PRIMENA TESTA DIREKTNOG KONTAKTA U ISPITIVANJU CITOTOKSIČNOSTI STOMATOPROTETSKIH AKRILATA

Milena Kostić, Stevo Najman, Jelena Najdanović, Nebojša Krunić i Ivan Kostić

Upotreba akrilata široko je rasprostranjena u stomatološkoj praksi. Pošto u ustima pacijenta imaju ulogu morfološkog i funkcionalnog supstituenta, akrilati se svrstavaju u grupu biomaterijala. Sa druge strane, klinička praksa pokazuje da pojedini toksični sastojci akrilata mogu dovesti do neželjenih promena lokalnog, a znatno ređe i sistemskog karaktera. Cilj istraživanja bio je ispitivanje citotoksičnog efekta različitih akrilatnih stomatoprotetskih materijala testom direktnog kontakta sa ćelijskom kulturom. Ispitivan je efekat četiri različite vrste akrilatnih materijala na HeLa ćelijsku kulturu. Nakon svetlosno mikroskopske analize urađen je MTT test, bez prethodnog uklanjanja uzoraka materijala. Dobijene vrednosti MTT-a ukazuju na zavisnost ćelijske proliferacije od vrste akrilatnog stomatoprotetskog materijala. Hladno polimerizovani akrilati pokazali su blagi infibitorni efekat na rast ćelijske kulture. U slučaju toplo polimerizovanog akrilata nisu opservirani znaci toksičnosti. *Acta Medica Medianae 2012;51(1):66-72.*

***Ključne reči**: akrilati, test direktog kontakta, HeLa ćelije*