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Original Article

Cytotoxicity of polymethylmethacrylate copolymers

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Abstract

Aim: This study aimed to determine the cytotoxicity of polymethylmethacrylate copolymers.

Material and Methods: The polymethylmethacrylate resin was used to fabricate the samples. Ethyl-methacrylate, butyl-methacrylate and isobutyl-methacrylate, were used to form the copolymerization of polymethylmethacrylate. 10%-20%-30%-40% ethyl-methacrylate, butyl-methacrylate and isobutyl-methacrylate monomers were added to the methyl-methacrylate, which is the monomer of the control group, and 13 different groups were formed. Five specimens (n=5) for cytotoxicity test were prepared for each group. Cytotoxicity effects of the resins at 24th and 48th hours were evaluated by MTT assay. The data of the cytotoxicity test was analyzed by applying one-way variance analysis.

Results: The effect of monomer type and monomer percent on cell viability was significant in both periods ($p< 0.01$). The percentage of cell viability of all groups at both times was over 90%. The cell viability showed a tendency to decrease by increasing the percentage of monomer added in both periods. The highest and lowest cell viability in 24th hours were observed in 10% ethyl-methacrylate and 30% butyl-methacrylate groups respectively, while the highest and lowest cell viability in a 48th hour was observed in 10% butyl-methacrylate and 40% butyl-methacrylate groups, respectively.

Conclusion: The cytotoxic effects of copolymer resins were not observed at 24th and 48th hours. Cell viability was found at more than 90% in both periods. It was shown that the copolymer resins formed by the addition of 10%, 20%, 30%, and 40% by volume of isobutyl-methacrylate, butyl-methacrylate, and ethyl-methacrylate to the methyl-methacrylate were biocompatible materials.

Keywords: Acrylic resins; cell survival; cytotoxicity tests, immunologic; denture bases; polymers.

INTRODUCTION

Polymers play a significant role in the dentistry. Acrylic resin based on polymethylmethacrylate (PMMA) is the most widely used denture base material (1). However, due to its failure of the mechanical properties, lack of resistance to fractures, PMMA is far from an ideal denture base resin. The reason for the fracture of the denture base may be due to the low fracture strength (2,3).

It has been attempted to improve the mechanical properties of PMMA by chemical modification or by adding material into the resin (4-7). However, an entirely satisfactory alternative material to PMMA has not been developed yet.

The use of two different chemically monomers are often an advantage in improving the physical properties of the resin while obtaining the denture base resin. The polymer thus obtained is called copolymer, and the term copolymerization is used for this process (8). Copolymer addition was used to strengthen the denture base (4). Due to the different polymers mixed in varying volumetric proportions, a new material with better properties may be obtained (9). It is crucial that the monomers forming the blend are compatible with one another. However, if the polymer pairs do not interfere with one another, a material with weaker mechanical properties may be formed (9). Monomers which are compatible with one another are known to form copolymers with improved mechanical properties (10,11). In this way, flexural strength, flexural modulus, impact strength, thermal durability, and adhesion properties may be improved (12). Compared to linear polymers, copolymers exhibit higher polymerization rates, increased mechanical properties, and lower water absorption properties (13).

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Hayran et al.(14) showed that the copolymerization of PMMA with ethyl methacrylate (EMA), butyl methacrylate (BMA) and isobutyl methacrylate (IBMA) monomers tended to increase the flexural strength and flexural modulus of the PMMA resin. However, a denture based resin is desired to be biocompatible in addition to excellent physical and mechanical properties. The texture surrounding the synthetic material and the absence of reaction in the body are characterized by the biocompatibility of the material (15). Allergic reactions and local irritation of acrylic denture base resins have been reported in many studies (16, 17). The purpose of this study was to determine the effect of PMMA copolymerization by adding EMA, BMA, IBMA monomers to methyl methacrylate (MMA) monomer on the cytotoxicity effects of the resins.

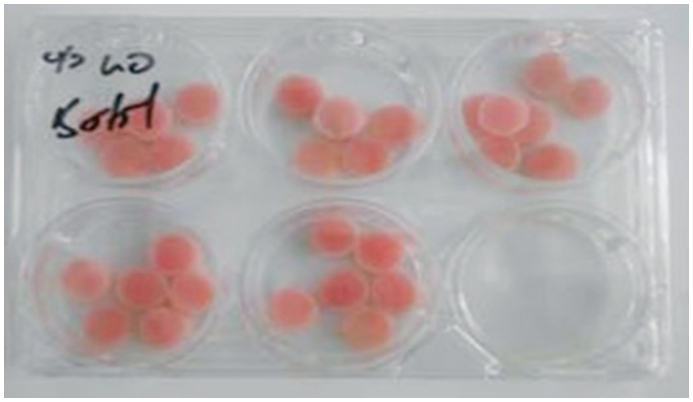

Figure 1. Disk specimens prepared for a group

MATERIAL and METHODS

Sample Preparation

The heat polymerized PMMA based resin was used to fabricate the samples. (QC-20, Dentsply Co, New Zealand, Australia). Three different monomers, EMA (Fluka, Sigma Aldrich GmbH, Germany), BMA (Fluka, Sigma Aldrich GmbH, Germany) and IBMA (Fluka, Sigma Aldrich GmbH, Germany), were used to form the copolymerization of PMMA. 10%-20%-30%-40% EMA, BMA, and IBMA monomers were added to the MMA, which is the monomer of the control group, and 13 different groups were formed. The monomer-polymer ratio was prepared by the manufacturer's instructions, and it was 23g/10 ml. Polymerization of the samples was carried out in a water bath for 30 minutes at 100°C according to manufacturers' instructions. The monomers and their volume percentage used in the study are presented in Table 1.

The number of samples for the MTT cytotoxicity assay was determined according to ISO 10993-12 (18). Five specimens (n=5) for cytotoxicity test were prepared for each group. In accordance with the specified values for the surface area of the material to be tested in the standard, 7 piece of polymer discs with a diameter of 10 mm and a thickness of 1.5 mm were prepared for one specimen of each group (Figure 1) (18). In this case, a total of 35 discs were prepared for each group. Stainless steel molds were used to obtain wax duplicates of test resin specimens according to ISO standards for cytotoxicity tests (18). After polymerization, the flasks were allowed to cool. All resins were then sanded with 600 grit sandpaper.

The cytotoxicity test specimens were sterilized in an autoclave before the (tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test. The ratio of the disc specimen's surface area to the extraction volume was adjusted to 3 cm^2 mL^{-1}, following ISO standard. The specimens were placed in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and incubated at 37°C in an atmosphere of 5% CO_2 in the air without agitation for 24, 48 hours periods. After 24 hours and 48 hour incubation times were completed, extracts were used to assess cytotoxicity.

Table 1. Volumetric percents of monomers

	100% MMA (Control)	
10% EMA+90% MMA	10% BMA+90% MMA	10% IBMA+90% MMA
20% EMA+80% MMA	20% BMA+80% MMA	20% IBMA+80% MMA
30% EMA+70% MMA	30% BMA+70% MMA	30% IBMA+70% MMA
40% EMA+60% MMA	40% BMA+60% MMA	40% IBMA+60% MMA

Cells

L-929 mouse fibroblast cells (HUKUK, Ankara, Turkey) were grown as monolayer culture using DMEM with 10% FBS at week at 37°C in an atmosphere of 5 % CO_2 in the air and 100% relative humidity. The cells were detached from the surface of T25 flasks using enzyme solution (0.025%) trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) incubated for 2–5 min at 37°C. After trypsinization, the cell suspension was prepared for MTT assay.

The Cytotoxicity Test

The L-929 cell suspension was prepared at a concentration of 3 x10^4 cells mL^{-1} by using DMEM with 10% FBS and dispensed onto 96-well cell culture plates as 100 µL per well. The multiwell plates were left to at 37°C, 5% CO_2 in the air and 100% relative humidity for 24-hour incubation. After 24 hours, the culture medium was removed from the wells, and 100 µl of the extracts were added into each well. In control wells, 100 µL fresh DMEM with 10% FBS was added. After the 24 hours and 48 hour incubation period at 37°C, 5% CO_2 in the air, plates were emptied and fresh DMEM and MTT dye solution were added to the wells left in a dark environment for 4 h at 37°C. The plates were evacuated and 100 µl isopropanol was added to dissolve the formazan crystals. Plates were mixed gently on a shaker to enhance dissolution. The absorbance of color developed was measured on an ELISA plate reader at 570 nm. Experiments were repeated three times throughout this study. The results were calculated as a percentage of the control group values. The cytotoxicity data were analyzed by applying the one-way ANOVA. Advanced statistical analyzes were performed by Duncan test. The numerical values of cell viability obtained by the MTT

test result were confirmed by examining the cells with an inverted tissue culture microscope (Olympus CK40, Tokyo, Japan).

RESULTS

According to the results of one-way ANOVA, the interaction between time and groups at 24th hours and 48th hours was statistically significant (p<0.01). The differences between the times for each group and the differences between the groups at each time were compared using the Duncan test. Means and standard error of means of cytotoxicity tests are shown in Table 2.

Comparison of the cell viability of the copolymer resin groups at 24th hours

According to the results of Duncan test, the 10% BMA group (92.291%), 20% BMA group (92.343%), 10% IBMA group (94.1%) and 20% IBMA (93.121%) group were not differ from the control group (93.123%) according to cell viability percentage at 24th hours (p>0.01), while the difference between the other copolymer groups and the control group was statistically significant (p<0.01). The cell viability percentage of the EMA groups was (95.552%-97.355%), the cell viability percentage of the BMA groups was (90.392%-92.343%), and the cell viability percentage of the IBMA groups was (91.251%-95.427%), respectively. The highest and lowest cell viability in 24th hours were observed in 10% EMA group (97.355%) and 30% BMA (90.392%) group, respectively.

The difference between the 10% EMA group and the 20% EMA group, and 30% EMA group was not statistically significant (p>0.01), but the difference between the other copolymer groups was significant (p<0.01). The difference between the 20% EMA group and all of the EMA groups and 40% IBMA group was statistically insignificant (p>0.01). There was no difference between the 30% EMA group and all of the EMA groups (p>0.01), but differed from all BMA groups and IBMA groups (p<0.01). The 40% EMA group did not differ from the 20% EMA group, 30% EMA group, 10% IBMA group and 40% IBMA group (p>0.01). The 10% BMA group and the 20% BMA group did not differ from the 30% BMA group, 20% IBMA group, 30% IBMA group (p>0.01). The 30% BMA group differed from the other copolymer groups except the 40% BMA group and the 30% IBMA group (p<0.01). The difference between 10% IBMA group and the other copolymer groups except 40% EMA group, 20% IBMA group and 40% IBMA group was significant(p<0.01). The 20% IBMA group was not statistically significant differences from 10% BMA group, 20% BMA group, and 10% IBMA copolymer group (p>0.01). The 30% IBMA group differed from the other copolymer groups except for all of the BMA groups (p<0.01). There was no difference between 40% IBMA group and 20% EMA group, 30% EMA group, 40% EMA group and 10% IBMA group (p>0.01).

The effect of monomer type and monomer percent on cell viability was significant (p< 0.01). The cell viability showed a tendency to decrease with increasing the percentage of monomer.

Comparison of the cell viability of the copolymer resin groups at 48th hours

When cell viability percentages at 48th hours were examined, 20% EMA (96.338%), 40% BMA (96.093%), 20% IBMA (96.715%) and 30% IBMA (95.06%) groups did not differ from the control group (95.213%), whereas other copolymer groups were statistically different from the control group. The cell viability percentage of the EMA groups was (96.338%-98.244%), the cell viability percentage of the BMA groups was (96.093%-99.497%), and the cell viability percentage of the IBMA groups was (93.511%-97.92%), respectively. The highest and lowest

Table 2: Cell viability (%) values of control and copolymer groups at 24th and 48th hours

Groups		n	Cell viability (%)	
			24th hour	48th hour
100% MMA (Control)		5	93.123 ± 0.366 **A de**	95.213 ± 0.426 **B ef**
EMA	10% EMA	5	97.355 ± 0.395 **A a**	98.244 ± 0.209 **A ab**
	20% EMA	5	96.716 ± 0.447 **A ab**	96.338 ± 0.303 **A cdef**
	30% EMA	5	95.916 ± 0.304 **A ab**	97.908 ± 0.384 **B abc**
	40% EMA	5	95.552 ± 0.406 **A bc**	97.753 ± 0.235 **B bc**
BMA	10% BMA	5	92.291 ± 0.590 **A ef**	99.497 ± 0.351 **B a**
	20% BMA	5	92.343 ± 0.259 **A ef**	98.224 ± 0.115 **B ab**
	30% BMA	5	90.392 ± 0.350 **A g**	96.807 ± 0.462 **B bcd**
	40% BMA	5	91.115 ± 0.334 **A fg**	96.093 ± 0.302 **B def**
IBMA	10% IBMA	5	94.100 ± 0.419 **A cd**	97.920 ± 0.522 **B bc**
	20% IBMA	5	93.121 ± 0.437 **A de**	96.715 ± 0.426 **B bcde**
	30% IBMA	5	91.251 ± 0.373 **A fg**	95.060 ± 0.819 **B f**
	40% IBMA	5	95.427 ± 0.126 **A bc**	93.511 ± 0.229 **B g**

One-way ANOVA was used. Advanced statistical analyzes were performed by Duncan test
* Capital letters were used to compare times in each group, p <0.01
* Lower case letters are used for comparison of groups at times, p <0.01
* Different letters indicate difference with statistical significance

cell viability in a 48th hour were observed in 10% BMA (99.497%) and 40% BMA (93.511%) groups, respectively.

The difference between the 10% EMA copolymer group and 20% EMA group, 10% BMA group, 40% BMA group, 30% IBMA group and 40% IBMA group was statistically significant (p<0.01). The difference between 20% EMA group and 10% EMA group, 10% BMA group, 20% BMA group and 40% IBMA group was found to be significant (p<0.01). The 30% EMA group differed only from the 40% BMA group and the 30% IBMA group (p<0.01). The difference between 40% EMA group and 10% BMA group, 40% BMA group, 30% IBMA group and 40% IBMA group was found to be significant (p<0.01). There was no significant difference between 10% BMA group and 10% EMA group, 30% EMA group and 20% BMA group (p>0.01). There was significant difference between 20% BMA group and 20% EMA group, 40% BMA group, 30% IBMA group and 40% IBMA group (p<0.01).The 30% BMA group differed from other copolymer groups except 10% BMA group, 30% IBMA group and 40% IBMA group (p<0.01). The difference between 40% BMA group and 20% EMA group, 30% BMA group, 20% IBMA group and 30% IBMA group was not significant (p>0.05). The difference between 10% IBMA group and 10% BMA group, 40% BMA group, 30% IBMA group and 40% IBMA group was significant (p<0.01). The difference between 20% IBMA group and 10% BMA group, 30% IBMA group, and 40% IBMA group was found to be significant (p<0.01). The 30% IBMA group differed from other copolymer groups except 10% BMA group, 20% EMA group and 40% BMA group (p<0.01). The 40% IBMA group was statistically different from all of the copolymer groups (p<0.01).

The effect of monomer type and monomer percent on cell viability was significant (p< 0.01). The cell viability showed a tendency to decrease with increasing the percentage of monomer.

Comparison of the cell viability according to the resin types at 24th and 48th hours

The effect of time on cell viability was statistically significant, except control group, 10% EMA group and 20% EMA group (p<0.01). When cell viability percentages were compared at 24th and 48th hours, the percentage of cell viability increased with time except for 20% EMA group and 40% IBMA group. The percentage of cell viability of all groups at both times was over 90%. The cell viability percentages ranged from 90.392% to 97.355% at 24th hours, while cell viability percentages ranged from 93.511% to 99.497% at 48th hours. There was no difference in the percentage of cell viability at 24th hours between the control group and 10% BMA group, 20% BMA group, and 20% IBMA group (p> 0.01). There was no difference in the percentage of cell viability at 48th hours between the control group and 20% EMA group, 40% BMA group, 20% IBMA group, and 30% IBMA group (p> 0.01).

Evaluation of cell viability with an inverted tissue culture microscope

In the regular appearance of the fibroblast cell, the cells are smooth and spindle-shaped. In the microscopic images of the cell control group, it is seen that the edge contours of the cells are bright, and the membrane structure is intact. Depending on the cytotoxicity, some changes occur in the cell structure. Findings are showing toxicity; rounding of cells, separation from the surface, a morphological disorder of cell structures, and the presence of giant cell structures. The cytotoxic changes in the cell structures of the control and copolymer groups at 24th and 48th hours are shown in Figure 2.

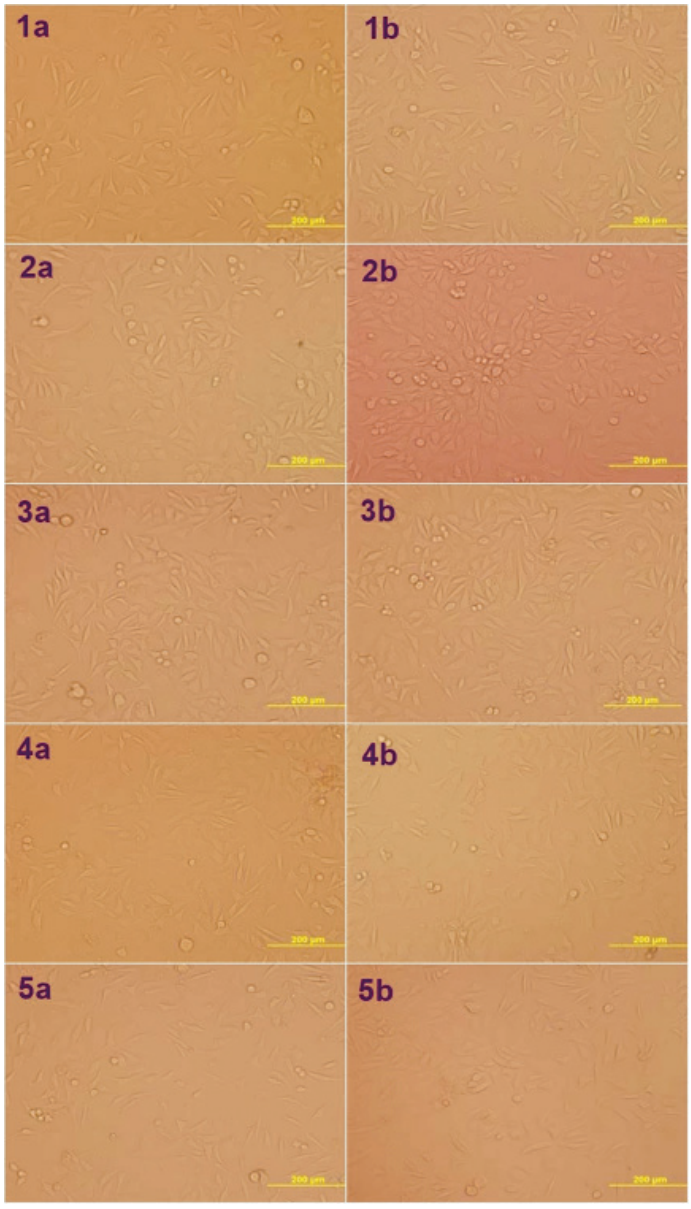

Figure 2. Microscopic views of the cells of the control and copolymer groups

Microscopic view of the cell control at (1a) 24th and (1b) 48th hour (x 200),
Microscopic view of the cells of the control group at (2a) 24th and (2b) 48th hours (x 200),
Microscopic view of the cells of the 30% EMA group at (3a) 24th and (3b) 48th hour (x 200),
Microscopic view of the cells of the 30% BMA group at (4a) 24th and (4b) 48th hour (x 200),
Microscopic view of the cells of the 30% IBMA group at (5a) 24th and (5b) 48th hour (x 200).

DISCUSSION

Several methods have been used to strengthen PMMA. However, an alternative material to PMMA has not been presented yet when it is evaluated together with its ease of production, stability, aesthetics and low price and despite all the negative features such as low strength, the most commonly used denture base material is PMMA (2). One of the methods for reinforcement of PMMA is to create a modification of PMMA by copolymerization. Copolymerization mechanism increases the amount of the polymerization in the polymer network, and this increase simultaneously increases the mechanical properties of the polymer. The cross-linked polymer network formed is insoluble in water and decreases the swelling of the resin (13). However, the biocompatibility of a material is very important in terms of clinical availability. If a denture base material is not biocompatible, it cannot be used even if its durability is excellent. The material in contact with the tissue should not form a local or systemic reaction in the body. Before it is widely used in clinical practice, dental materials need to be approved by standards not only for mechanic properties but also for biological properties.

This study aimed to determine the effect of copolymerization of a conventional heat polymerized acrylic resin with the participation of EMA, BMA, and IBMA into the monomer of PMMA (MMA) on the biological properties of PMMA. The ratios of EMA, BMA, and IBMA monomers were chosen to be 10%, 20%, 30% and 40%, so that the main monomer, MMA, was dominant in the copolymer while strengthening the polymer structure. When the literature was evaluated, various methods were used for cytotoxicity analysis. The most commonly used cytotoxic test method is the enzymatic MTT test that measures mitochondrial dehydrogenase activity (19-22). The mitochondrial dehydrogenase enzyme is separated from the active mitochondria in living cells and converts the pale yellow tetrazolium salt, MTT ([3-(4,5–dimetiltiazol-2-il)-2,5-diphenyl tetrazolium bromide], into a dark blue insoluble formazan (1-[4,5- dimetil-tiazol-2-il]-3,5-difenilformazan) compound (23). The production of mitochondrial dehydrogenase determines live cell count. In this study, the cytotoxic effects of copolymer resins at 24 and 48 hours on L-929 mouse fibroblasts were investigated by MTT analysis. In cell cultures, L-929 (24) and 3T3 mouse fibroblasts (25) or primary cells (25) such as gingiva, mucosa, and pulp fibroblasts are used (26). Therefore, L929 mouse fibroblast was used in the study.

During the polymerization of the acrylic resins, the conversion of the monomer to the polymer is not complete, and the various amounts of free and unreacted monomer remain in the polymerized resin (27). The most important factor affecting the cytotoxic potential and biocompatibility of acrylic resins is the released monomer (28). Unreacted monomers can be released from polymerized acrylic resin, and this may irritate the mucosa. Potentially toxic substances, such as formaldehyde, methyl methacrylate, methacrylic acid, benzoic acid, dibutyl phthalate, phenyl benzoate, phenyl salicylate, and dichlohylfluorate, can be released from the prosthetic base resin (29). At the same time, the monomer acts as a plasticizer and negatively affects the physical and mechanical properties of acrylic resins. It has been shown that substances which can be released from acrylic resin have a cytotoxic potential at the rate of release (30,31). It is desirable that the residual monomer level in the acrylic prosthesis base is placed in the mouth as low as possible. In addition to the durability, aesthetics, and function of the dental materials, it is also important to be biocompatible. Many factors, such as polymer-monomer ratio, polymerization time, polymerization method, water immersion after polymerization of the resins, are active on the cytotoxicity of the resin (31,32).

Polymer-monomer ratio is one of the factors affecting the cytotoxicity of the denture base resin. It is thought that the addition of more monomers to the mixture increases the residual monomer content of the resin and therefore the resin may have a more significant cytotoxicity potential. In the present study, the monomer-polymer ratio was prepared by the manufacturer's instructions, and it was 23g/10 ml.

The polymerization temperature for the residual amount of monomer is also critical. In the studies performed, it has been shown that more MMA remains in the polymers than the boiling at temperatures over 100°C in case of boiling at temperatures lower than 100°C (31). In the present study, polymerization was carried out in boiling water at 100°C.

In this study, 10993-5(33) and 10993-12(18) standards prepared by ISO have been taken into consideration in the investigation of the cytotoxicity of the copolymer structures. According to the evaluation of cytotoxicity of dental materials of ISO 10993-5 (33) ; A cell inhibition of less than 25% indicates that the material is not cytotoxic (Grade 0), cell inhibition between 25-50% indicates that the material is mild cytotoxic (Grade 1), 50-75% cell inhibition indicates that the material is moderate cytotoxic (Grade 2) and cell inhibition more than 75% indicates that the material is highly cytotoxic (Grade 3). According to the results of this study, no cytotoxic effect was observed in the copolymer groups at 24th and 48th hours incubation period. The cell viability was higher than 90% in all groups, including the control group at 24th and 48th hours. It was observed that cell viability in 48th h was generally higher than 24th hours for all resin groups. When the studies on the cytotoxic effects of denture base resins were examined, it has been shown that the cell viability increases with time as it confirms the present study (29,34,35). The most important factor affecting the cytotoxic potential and biocompatibility of acrylic denture base resins is the residual monomer released (31,36,37). Sheridan et al. (35) reported that the cytotoxic effects of acrylic resins were very high in the first 24 hours after polymerization, and this effect decreased over time. It is thought that the

reason for the cell viability at 24th hours was less than the 48th hours, because the residual monomer released from the resin in the first 24 hours may be more.

It has seen that mechanical, physical, and thermal properties of various denture base resins produced from copolymer were generally examined in the literature. To the best of our knowledge, there was only one study similar to our study in which the cytotoxic effects of the formed copolymer resin are examined (6). In the mentioned study, 2%, 3%, and 5% IBMA monomer ratios were added into PMMA in order to perform the copolymerization. As in the present study, no cytotoxicity was observed in the copolymer structures.

In previous studies of Hayran et al.(14), these synthesized copolymers were confirmed both FTIR and NMR spectroscopy. Although Hayran et al.(14) showed that the copolymerization of PMMA with ethyl methacrylate (EMA), butyl methacrylate (BMA) and isobutyl methacrylate (IBMA) monomers tended to increase the flexural strength and flexural modulus of the PMMA resin. The present study synthesized EMA, BMA, and IBMA copolymers of PMMA and evaluated the cytotoxicity as biological properties. It was shown that the copolymer resins formed by the addition of 10%, 20%, 30%, 40% by volume of IBMA, BMA, and EMA to the MMA monomer were biocompatible materials.

Nonetheless, there are limitations to the present study. Firstly in the present study, cytotoxicity was evaluated at 24th and 48th hours, and cytotoxicity should be evaluated for more extended periods. Secondly, the residual monomer release of the copolymer structures should be studied.

CONCLUSION

It was shown that the copolymer resins formed by the addition of 10%, 20%, 30%, 40% by volume of IBMA, BMA, and EMA to the MMA monomer were biocompatible materials. The cytotoxic effects of copolymer resins were not observed at 24th and 48th hours. Cell viability was found at more than 90% in both periods. The copolymer structures formed by copolymerization of PMMA with the addition of EMA, BMA, and IBMA to MMA are promising materials for denture base resin. These copolymer structures can be used safely according to the results of the present study.

Competing interests: The authors declare that they have no competing interest.

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