

EXPLORING COMPARATIVELY THE CYTOTOXICITY OF MILLED AND 3D-PRINTED PMMA-BASED RESINS TO ORAL SQUAMOUS CANCER CELLS - WHAT SIGNALS COULD THE AKT/MTOR PATHWAY SEND US THROUGH ITS DOWNSTREAM EFFECTOR GSK-3?

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Abstract

Aim of the study In this study, we aimed to provide more experimental data regarding the possible cytotoxic effects of two types of PMMA-based resin samples (CAD/CAM milled and 3D-printed) on human oral squamous carcinoma cells. Furthermore, we have also explored the possible alterations of the Akt pathway and its downstream target, apoptosis, in the exposed cells to the two types of resin samples. During the last few years, PMMA-based materials have been extensively explored and modified in order to obtain the desired and ideal properties for dentistry applications. However, there are certain unsolved issues regarding the PMMA-based obturators, including possible cytotoxicity due to the residual monomers. As far as we know there are no studies targeting the Akt/mTOR signalling pathway in the human oral squamous carcinoma cells, exposed to PMMA-based resins used for maxillary obturator manufacturing. **Material and methods** 25 samples of PMMA-based resin were printed with SHERAeco-print 30 resin using Digital Light Processing (DLP) technique. The resin was layered with a thickness of 50 µm. The Human Oral Squamous Carcinoma Cell Line (OECM-1 cell line) were cultured in a humidified atmosphere at 37 degrees Celsius and 5% carbon dioxide. **Results** Our results showed that the exposure of OECM-1 cells to two types of PMMA-based dental resins induced cytotoxic effects, probably via a residual monomer-induced overproduction of ROS, triggering the decrement of cell viability. Furthermore, exposure to the PMMA-based resins disturbed the Akt/mTOR/GSK-3 signaling pathway compared to the unexposed control cells. Moreover, our results might suggest that the noticed decreased cell viability should be attributed to the activation of apoptosis, via a detouring signaling pathway, possibly orchestrated by Bcl-2. **Conclusion** Within the limitations of the present in vitro study, our results surprisingly revealed that exposure of OECM-1 cells to the two types of PMMA-based dental resins (MA-CAD/CAM and MA-3D printed) induced cytotoxic effects and reduced cell viability probably by activation of apoptosis. Due to the experimental limitations of the present study, our data must be considered preliminary.

Key words: PMMA-based, dental resin, Akt/mTor, GSK3, apoptosis, maxillary obturator

Introduction

Clinical dentistry commonly uses a wide range of polymeric materials for various applications (1,2). One of the most used polymers in dentistry is the polymethyl-methacrylate (PMMA). PMMA deserves its popularity due to its unique physical and

mechanical properties (3-6). However, in the dentistry context, there still are several concerns regarding PMMA applicability. Oral healthcare applications of PMMA-based resins include temporary crowns, occlusal splints, denture relining, artificial teeth and maxillary obturator prosthesis.

The obturator is a special prosthesis designed to replace the lost maxillary tissue, in order to restore the patient's functional capabilities (such as deglutition, mastication, speech and aesthetics), after a maxillectomy (7,8). Despite the numerous studies carried out in dental materials science, presently there are only a few material options for obturators manufacturing (8). PMMA-based resins are the most commonly selected materials for maxillary obturators (8). During the last few years, PMMA-based materials have been extensively explored and modified in order to obtain the desired and ideal properties for dentistry applications (9). However, there are certain unsolved issues regarding the PMMA-based obturators, including polymerization shrinkage, difficulties with the undercuts due to the rigidity of the material and, last but not least, possible cytotoxicity due to the residual monomers (10).

Presently, PMMA-based resins are still considered a true „gold standard” material for maxillary obturators and other oral splints. However, the conventional oral splint manufacturing is increasingly being replaced by computer-aided designing and computer-aided manufacturing (CAD/CAM) techniques (11). The two commonly used CAD/CAM techniques for oral splint manufacturing are milling and 3D printing. Milling represents a subtractive process, in which the oral splint is carved from a prefabricated polymeric resin block (12). Unlike the milling method, 3D printing is an additive process during which liquid monomers are polymerized, generating the designed solid oral splint (13).

PMMA, IUPAC name poly [1-(methoxy carbonyl)-1-methyl ethylene], is a polymer synthesised by the free radical addition of methyl methacrylate to polymethyl methacrylate (9). The polymerization reaction is activated and initiated by a free radical generation using energy such as light, heat or microwaves (9). However,

because the monomer-to-polymer addition during polymerization reaction is incomplete, the residual unpolymerized monomers are released from the polymer matrix, interfering with the adjacent oral cells' functions (14).

Numerous previous studies outlined the adverse effects and cytotoxicity of dental PMMA-based resins at both cellular and tissue levels. However, very few studies targeted the deeper molecular level, more precisely, the signalling pathways (15-19). Studies conducted on permanent cell lines or primary cell cultures developed from gingiva, periodontium or dental pulp, revealed that both PMMA-based resins and the MMA monomers induced cytotoxic effects translated at the cellular level by autophagy-controlled apoptosis induction, cell cycle delay, mineralization inhibition (15-19). Many studies strongly highlighted that the discrete molecular event that underlies these cytotoxic effects involves the oxidative stress generation (19). It has been outlined that cells' exposure to MMA and related monomers, induced the decrease of the most important cellular antioxidant, glutathione, triggering at the same time, excessive reactive oxygen species (ROS) production, and finally leading to oxidative stress generation (19). One of the most important actors in the oxidative stress-controlling scene is the Akt/mTOR signalling pathway (20). The activated Akt is extremely important for cell survival. ROS are necessary for the activation of Akt/mTOR signalling, which in turn, will regulate the cellular redox homeostasis, very important for cell growth and proliferation (14). Moreover, the Akt pathway represents a key signalling pathway that regulates autophagy, apoptosis, cell proliferation and the molecular mechanisms underlying metastasis (20). Recent studies outlined that Akt pathway was the most frequently upregulated pathway triggering tumorigenesis and tumor development (21). Moreover, the AKT/mTOR pathway is

considered the main pathway involved in the initiation and regulation of autophagy. The inhibition of this pathway enhanced the signal of autophagy activation (22,23). Moreover, an increasing number of studies highlight the deep involvement of the Akt pathway in oral squamous cell carcinoma (OSCC) (14). Accordingly, in this study, we aimed to provide more experimental data regarding the possible cytotoxic effects of two types of PMMA-based resin samples (CAD/CAM milled and 3D-printed) on human oral squamous carcinoma cells. Furthermore, we have also explored the possible alterations of the Akt pathway and its downstream target, apoptosis, in the exposed cells to the two types of resin samples. As far as we know there are no studies targeting the Akt/mTOR signalling pathway in the human oral squamous carcinoma cells, exposed to PMMA-based resins used for maxillary obturator manufacturing. The null hypothesis tested was that exposure to the two types of PMMA resin samples would induce no cytotoxic reactions in the tested cells and no effects on the Akt/mTOR signalling pathway, nor on the apoptotic pathway.

Materials and methods

1. Additive manufacturing (3D printing)

The 25 samples were printed with SHERAeco-print 30 resin using Digital Light Processing (DLP) technique. The resin was layered with a thickness of 50 μm . The angulation of 20° to the building platform was set. The cleaning and the post-polymerization step were done using SHERAclean-p, SHERAultra-p and SHERAflash-light plus followed the instructions as described in literature (24).

2. Subtracting manufacturing

The 25 discoidal samples were designed using CAD/CAM software. Discs with a diameter of 12 $\pm$ 2 mm and 3 $\pm$ 1 mm were milled from industrially prefabricated disc PM20 blanks using the CORiTEC 350i innovative machine.

3. Specimens finalisation

The circular surface of the fabricated discs was high gloss polished according to a set polishing protocol, using Abraso Star Glaze and Polirapid a cotton cloth fine disc from Germany. This meant grinding with corundum sandpaper, rubberizing with 3-stage polishers for acrylics, pro-polishing with a synthetic brush, and pumice powder. Afterwards, the specimens were cleaned in a steam jet and ultrasonic bath.

4. Cell Culture

The Human Oral Squamous Carcinoma Cell Line (OECM-1 cell line) were cultured in a humidified atmosphere at 37 °C and 5% carbon dioxide. Complete RPMI 1640 medium supplemented with 5% FBS and 1% penicillin/streptomycin/amphotericin was used to culture the cells. The tumoral cells were seeded at a cell density of 2 x 10⁵ cells/well and cultivated overnight on 24-well. Then the tumoral cells were exposed to two types of sterilized dental material samples: MA-based CAD/CAM and MA-based 3D printed for the next 24 hours at 37°C with 5% CO₂. Subsequent of the incubation time dental material samples were removed with sterile forceps. The control samples were represented by cells without the dental materials. The dental material samples were previously incubated with alcohol for 5 minutes. Further, sterilization continued for 30 minutes on each side under UV light.

5. Cell Viability Assay

The viability of the cells was determined using the MTT assay from Thermo Fisher Scientific, Oregon USA. The dental material samples and the growth medium were removed from wells after 24 hours of incubation, then 1000 μL of RPMI medium and 100 μL MTT was added to each well for 4 hours at 37°C. The formazan crystals were then solubilized with 1000 μL /well of isopropanol containing HCl (0.04 N) and the optical density was measured spectrophotometrically at 570 nm with a multi-mode microplate reader from BMG LABTECH (Ortenberg, Germany).

6. Griess Assay

The Nitric Oxide Assay Kit (NO, Thermo Fisher Scientific, Vienna, Austria) was used to quantify the concentration of nitric oxide (NO) from culture medium after 24 hours of incubation, NO is produced from reduced nitrites. The principle of the Nitric Oxide Assay Kit involves the reaction of nitrite with the Griess reagents, resulting in the formation of a pink product that can be measured spectrophotometrically at 540 nm.

7. Lactate Dehydrogenase (LDH) Assay

LDH (lactate dehydrogenase) assay is based on the principle of measuring the activity of the enzyme lactate dehydrogenase. LDH is released in medium when the cellular membranes are damaged. Therefore, LDH Cytotoxicity Assay from Thermo Fisher Scientific was used to determine cytotoxicity after 24h of incubation with dental materials, as directed by the manufacturer. The assay involves the conversion of lactate to pyruvate in the presence of the enzyme lactate dehydrogenase, and the simultaneous conversion of NAD^+ to NADH. Absorbance was measured after 30 minutes as described in our previous study (25).

8. Cell lysis

In order to extract the proteins, OECM-1 cells were lysed in Cell Signaling Lysis Buffer from a Luminex kit (Akt/mTOR kit) provided by Merck, containing phosphatase inhibitors supplied with Protease Inhibitor Cocktail 1x (G6521, Promega). Protein concentrations were quantified using the Bradford procedure that employs the dye Brilliant Blue G using the Total Protein Kit, Micro (Sigma-Aldrich, cat no TP0100). The samples were aliquoted in Eppendorf tubes and stored at -80° Celsius degrees for subsequent Luminex analyses.

9. Luminex

MILLIPLEX MAP Akt/mTOR Phosphoprotein 11-plex Magnetic Bead and MILLIPLEX MAP 7-Plex Early Apoptosis Magnetic Bead Kits from Merck were used. Protein levels in each sample were normalized after performing protein assays

with the Bradford assay. The final concentration used for Luminex kits was 0.44 mg/ml, the samples being diluted in Milliplex Assay Buffer.

Firstly, 50 μL of Assay Buffer was added into each well on the 96-well plates and mixed on a plate shaker. After 10 minutes, Assay Buffer was discarded and 25 μL of 1X bead suspension were added in the plates. Then 25 μL of each samples were added to appropriate wells. After 18 hours of incubation at 4°C on a plate shaker, the wells were washed twice with 100 μL Assay Buffer per well. 25 μL /well of 1X MILLIPLEX® Detection Antibody were added for 1 hour, following by the same amount of streptavidin-phycoerythrin (SAPE) for 15 minutes and of MILLIPLEX® Amplification Buffer for another 15 minutes. Finally, the wells were rinsed with 150 μL of Milliplex Assay Buffer and the mean fluorescence intensity (MFI) data of assay plates were read and analyzed using the Luminex 200 system.

10. Statistical Analysis

Data from the study were analyzed using IBM SPSS Statistics 25 and illustrated using Microsoft Office Excel/Word 2021. Quantitative variables were tested for normal distribution using the Shapiro-Wilk Test and were written as means with standard deviations along with 95% confidence intervals for means. Quantitative variables with normal distribution were tested between groups using Student's T-Test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) /Welch T-Test (# $p < 0.05$) according to the equality of variances between groups measured using Levene's Test. Differences between groups were measured as mean differences with 95% confidence intervals. The $p < 0.05$ values were considered statistically significant.

Correlations between quantitative variables with normal distribution were calculated using Pearson's correlation coefficient while correlations between quantitative variables with non-parametric distribution

were calculated using the Spearman's rho correlation coefficient.

Results

1. Cell Viability Analysis

The results of the MTT assay revealed that the cell viability of OECM-1 tumoral cells was significantly decreased after 24 hours of exposure to both dental materials, MA-

CAD/CAM and MA-3D (Figure 1). In the case of MA-3D sample viability decreased the most by a percentage of approx. 17% compared to the control. This suggested that both materials had a negative impact on cell viability, therefore tested methacrylate-based dental materials have a cytotoxic effect on OECM-1 tumoral cells.

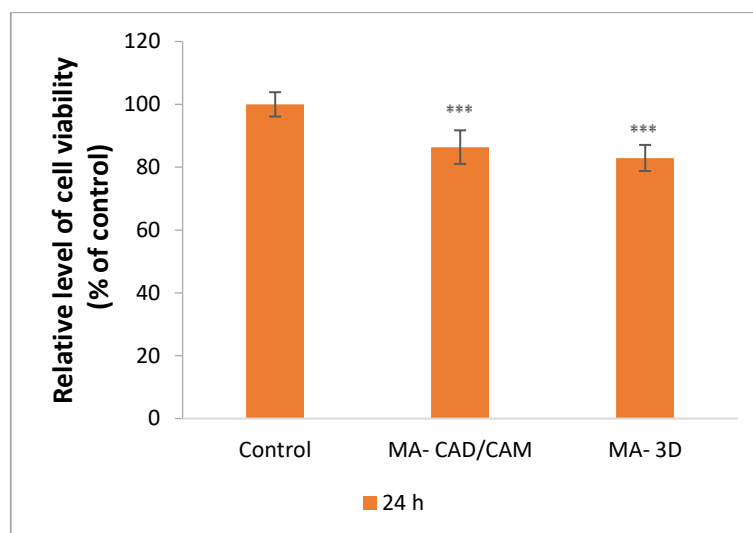


Figure 1. Effect of MA-CAD/CAM and MA-3D dental materials on OECM-1 tumoral cells viability after 24 hours of incubation, compared to control (unexposed cells). Results are means $\pm$ SD (n = 7). *** $p < 0.001$

Regarding the results of the Griess test, in the case of the MA-CAD/CAM samples, the level of NO was not changed compared to the control (Figure 2). On the other hand, in the case of MA-based materials obtained by the 3D technique, a significant increase in the level of NO was observed by approx. 16% after 24 hours of incubation, results correlated with the values obtained also after the LDH test. These findings showed that MA-3D have the potential to induce

nitric oxide production in tested oral tumoral cells, suggesting that the dental materials obtained by the 3D-printed technique have a potential pro-inflammatory effect on the tested oral squamous cancer cells. Overall, these results suggest that dental materials obtained by the 3D-printed technique may have a higher cytotoxic and pro-inflammatory effect on oral squamous cancer cells compared to the control.

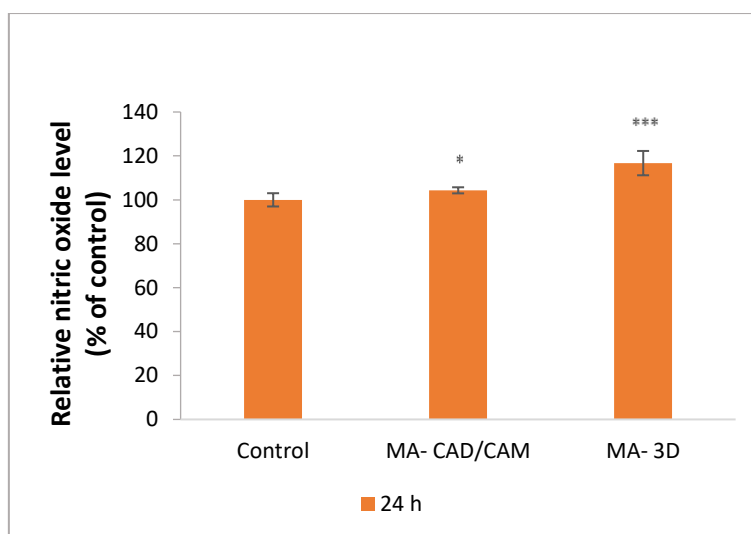


Figure 2. Effect of MA-CAD/CAM and MA-3D dental materials on OECM-1 tumoral cells on NO level after 24 hours of incubation, compared to control (unexposed cells). Results are means $\pm$ SD (n = 5). * $p < 0.05$, *** $p < 0.001$

Moreover, the results showed that after 24 hours of incubation, the levels of LDH in the OECM-1 tumoral cells were significantly increased in the presence of both MA-CAD/CAM and MA-3D compared to control cells, especially after the exposure of tumoral cells to MA-based

dental materials obtained by the 3D technique, it increased by 50% of control (Figure 3). The increase in LDH levels indicates cell membrane damage, suggesting a higher cytotoxic effect of the 3D-printed materials on the oral squamous cancer cells.

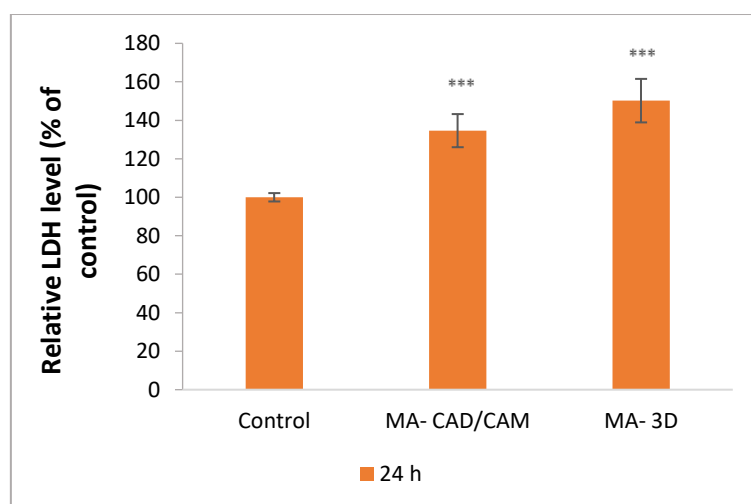


Figure 3. Effect of MA-CAD/CAM and MA-3D dental materials on OECM-1 tumoral cells on released LDH level after 24 hours of incubation, compared to control (unexposed cells). Results are means $\pm$ SD (n = 7). ** $p < 0.01$ and *** $p < 0.001$

Therefore, the results of LDH, MTT and Griess assays were correlated, regarding MA-based 3D samples.

2. GSK3 α and PTEN proteins' Expression Evaluation

The level of GSK3 α protein in OECM-1 tumoral cells after exposure to dental materials MA-CAD/CAM is presented in Figure 4. The results showed a higher concentration of GSK3 α protein after

exposure compared to control, increasing by 50% of control.

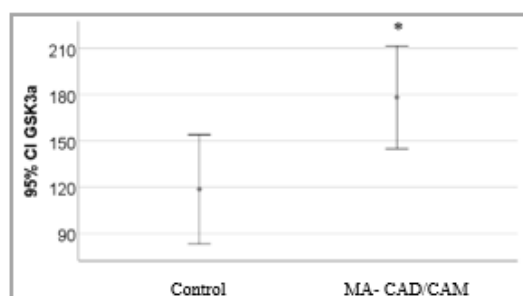


Figure 4. Effect of MA-CAD/CAM dental materials on OECM-1 tumoral cells on the level of protein GSK3a after 24 hours of incubation, compared to control (unexposed cells). Results are means $\pm$ SD (n = 6). * $p < 0.05$ compared to control.

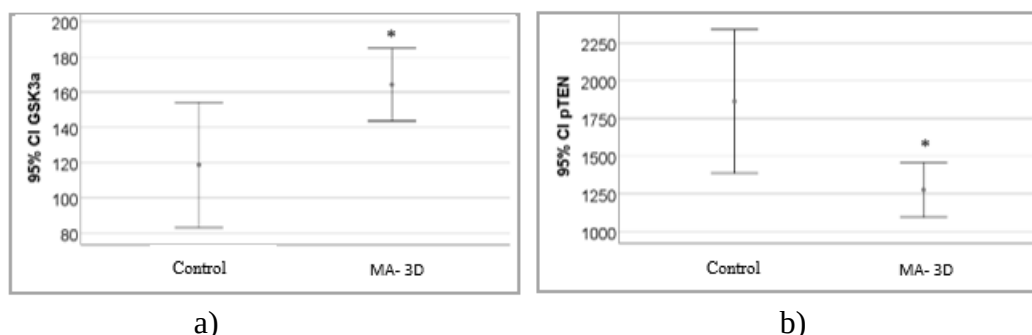


Figure 5. Effect of MA-3D dental materials on OECM-1 tumoral cells on the level of a) protein GSK3a and b) protein PTEN after 24 hours of incubation, compared to control (unexposed cells). Results are means $\pm$ SD (n = 6). * $p < 0.05$ compared to control.

In the case of incubation with MA-3D dental materials for 24 hours, the level of GSK3a protein expression (Figure 5 a) was increased by 38% compared to control. However, the level of pTEN decreased by 32% compared to control (Figure 5 b).

3. Early Apoptosis Evaluation

The distribution of all analyzed parameters was normal in both groups according to the Shapiro-Wilk Test ($p > 0.05$). The differences observed between groups were significant according to the Student T-Tests/Welch T-Tests for JNK, p53, Bcl-2, Caspase-9, and Caspase-8 proteins.

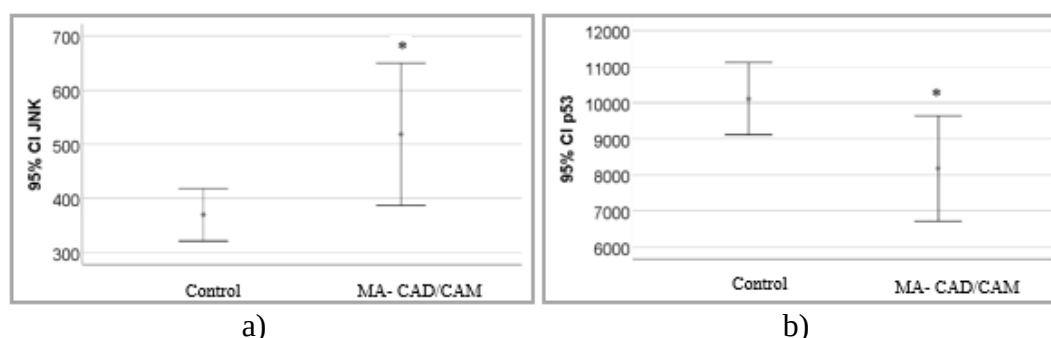


Figure 6. Effect of MA-CAD/CAM dental materials on OECM-1 tumoral cells on the level of a) protein JNK and b) protein p53 after 24 hours of incubation, compared to control (unexposed cells). Results are means $\pm$ SD (n = 6). * $p < 0.05$ compared to control.

After exposure of OECM-1 cells to MA-CAD/CAM dental materials for 24 hours, despite the fact that the levels of p53 protein were decreased in comparison with the control (Figure 6 b), the JNK protein expression was increased by 40% compared to control (Figure 6 a).

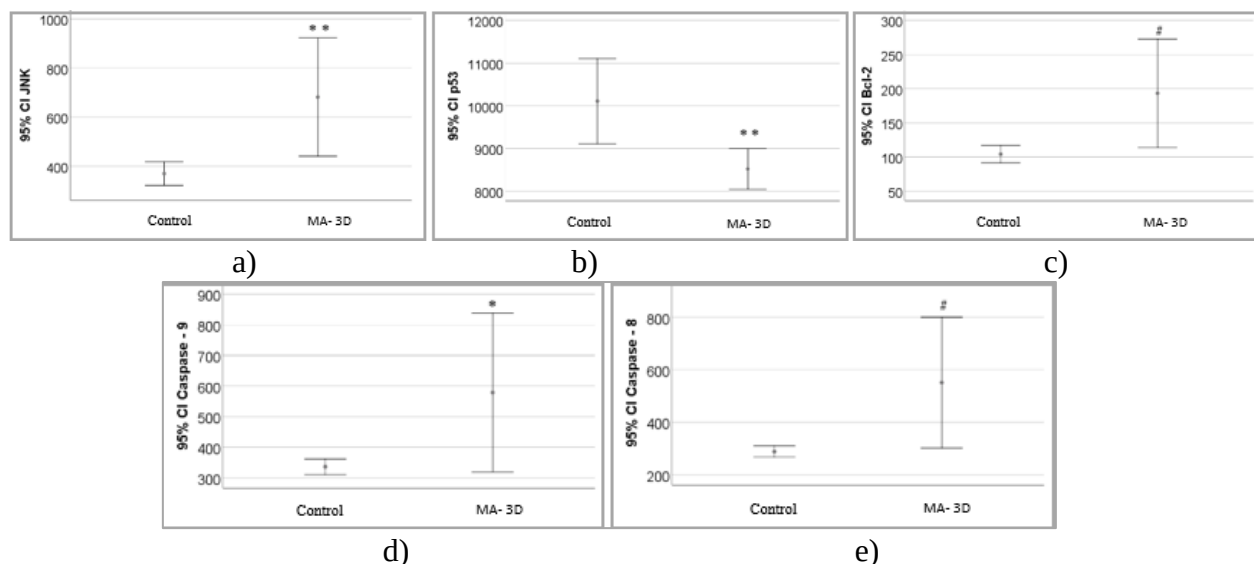


Figure 7. Effect of MA-3D dental materials on OECM-1 tumoral cells on the level of a) protein JNK, b) protein p53, c) protein Bcl-2, d) Caspase-9 and e) Caspase-8 after 24 hours of incubation, compared to control (unexposed cells). Results are means $\pm$ SD (n = 6). * $p < 0.05$ and # $p < 0.05$ compared to control.

An increased level of JNK, Bcl-2, Caspase-9 and Caspase-8 protein expression were revealed in cells incubated MA-3D after 24h incubation compared to control (Figure 7 a,b,c,d,e).

A significant increase in JNK and caspase Bcl-2 expression (by 84% and 85%, respectively, compared to the control) was induced following the exposure for 24 hours (Figure 7 a and c). In addition, the MA-3D

dental materials induced an increase by almost 72% of control in the caspase-9 expression (Figure 7 d). Furthermore, the caspase-8 level almost doubled (it increased by 91% of control) after exposure compared with the level of caspase-8 in the control (Figure 7 e). Regarding the p53 protein a 16% decrease in protein level from control was recorded after 24h.

Table 1. Correlations between analyzed parameters after 24 hours of incubation of OECM-1 tumoral cells in the presence of MA-CAD/CAM.

Correlation	p*
Bcl-2 (p=0.068**) x Caspase 9 (p=0.102**)	<0.001, R= 0.995
Bcl-2 (p=0.068**) x Caspase 8 (p=0.202**)	<0.001, R= 0.994

*Pearson Correlation Coefficient, **Shapiro-Wilk Test

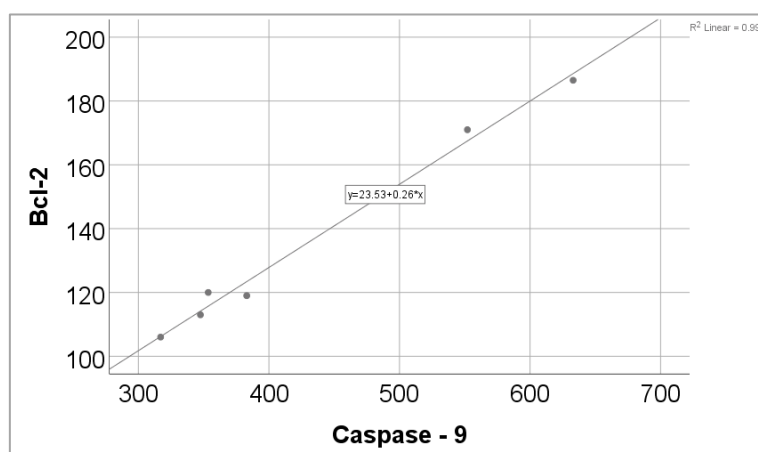


Figure 8. Correlation between Bcl-2 and Caspase-9 after 24 hours of incubation of OECM-1 tumoral cells in the presence of MA-CAD/CAM.

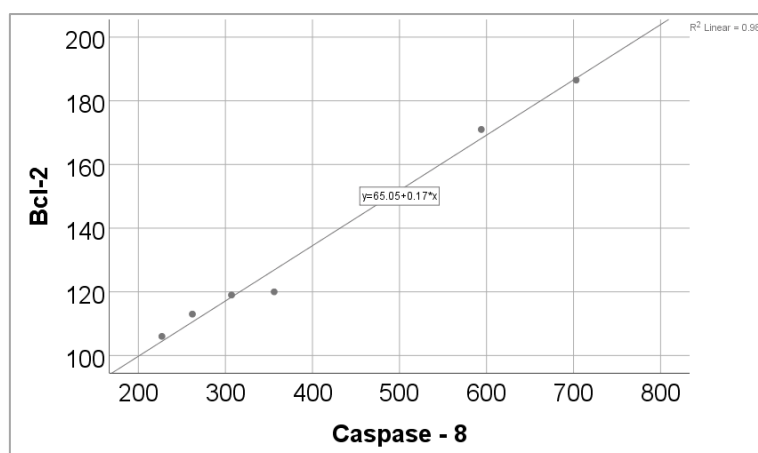


Figure 9. Correlation between Bcl-2 and Caspase-8 after 24 hours of incubation of OECM-1 tumoral cells in the presence of MA-CAD/CAM

Data from Table 1 and Figures 8, and 9 have shown the correlations between analyzed parameters after 24 hours of incubation of OECM-1 tumoral cells in the presence of MA-CAD/CAM. Significant correlations were observed only between Bcl-2 and Caspase-9 ($p < 0.001$, $R = 0.995$) and between Bcl-2 and Caspase-8 ($p < 0.001$, $R = 0.994$), indicating significant positive very high-power correlations which show that high values of Bcl-2 were significantly more associated with high values of Caspase-9 or Caspase-8 and vice versa.

Table 2. Correlations between analyzed parameters after 24 hours of incubation of OECM-1 tumoral cells in the presence of MA-3D.

Correlation	p*
Bcl-2 ($p=0.676^{**}$) x Caspase 9 ($p=0.520^{**}$)	0.007, R= 0.931
Bcl-2 ($p=0.676^{**}$) x Caspase 8 ($p=0.785^{**}$)	0.001, R= 0.981

*Pearson Correlation Coefficient, **Shapiro-Wilk Test

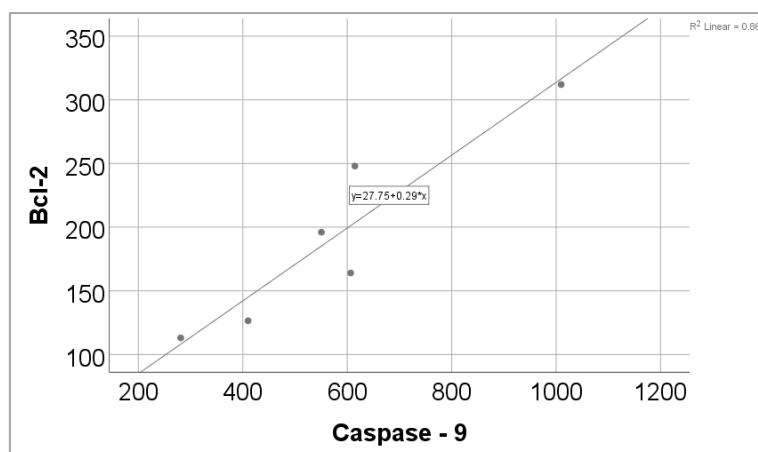


Figure 10. Correlation between Bcl-2 and Caspase-9 after 24 hours of incubation of OECM-1 tumoral cells in the presence of MA-3D.

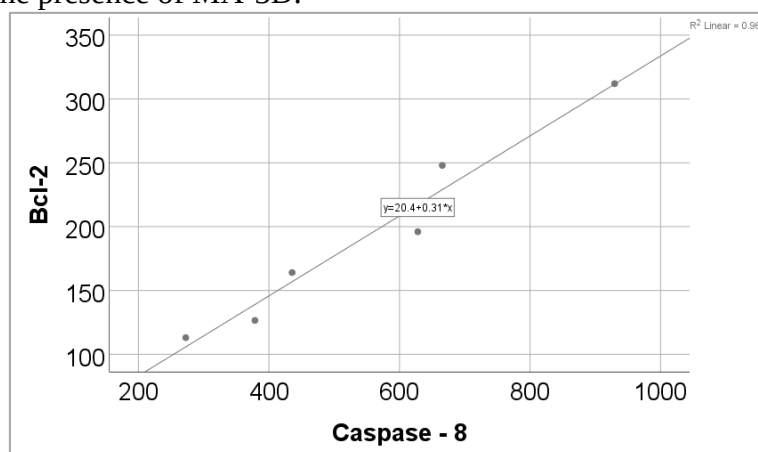


Figure 11. Correlation between Bcl-2 and Caspase-8 after 24 hours of incubation of OECM-1 tumoral cells in the presence of MA-3D.

Data from Table 2 and Figures 10, and 11 have shown the correlations between analyzed parameters after 24 hours of incubation of OECM-1 tumoral cells in the presence of MA-3D. Significant correlations were observed only between Bcl-2 and Caspase-9 ($p=0.007$, $R=0.931$) and between Bcl-2 and Caspase-8 ($p=0.001$, $R=0.981$), indicating significant positive very high-power correlations which show that high values of Bcl-2 were significantly more associated with high values of Caspase-9 or Caspase-8 and vice versa.

Discussion

In this study, the cytotoxic effect of milled and 3D-printed maxillary obturator resins, used on OECM-1 cells was explored.

Our MTT viability test results revealed that OECM-1 cells' viability was significantly decreased after 24 hours of exposure to both dental materials, MA-CAD/CAM and MA-3D (Figure 1). Moreover, in the case of the MA-3D sample, the viability decreased the most, by a percentage of approx. 17% compared to control. These results suggested that both materials might had a negative impact on the cell viability in the case of OECM-1 cells. These findings are also sustained by the LDH test results. Our experimental data showed that after 24 hours of incubation, the LDH levels in the OECM-1 tumoral cells were significantly increased in the presence of both MA-CAD/CAM and MA-3D compared to control. The greatest increase (50% of control) has been observed after the

exposure of tumoral cells to MA-based dental materials obtained by the 3D technique (Figure 3). The LDH levels increase outlined a possible cell membrane damage, suggesting a higher cytotoxic effect of the 3D-printed materials on the oral squamous cancer cells. Furthermore, interestingly, in the case of MA-based materials obtained by the 3D technique, a significant increase in the level of NO was observed by approx. 16% after 24 hours of incubation. These findings align with the results obtained for the LDH levels and viability tests. These results suggest that the MA-3D samples might have had a potential pro-inflammatory effect (illustrated by the increased NO levels) on the tested oral squamous cancer cells.

Many studies targeting PMMA-based resins' biocompatibility, highlighted that during the polymerization reaction the monomer conversion to polymer is never complete. Therefore, the inevitable consequence of these incomplete polymerization reactions is the accumulation of residual unreacted monomers and other chemical products, such as methyl methacrylate, methacrylic acid, benzoic acid, and formaldehyde within the polymeric matrix (24). All these chemical species, with low molecular weights, are able to leach into the saliva via a diffusion process and subsequently interact with the cells of the oral mucosa, inducing cytotoxic effects (24). These findings might explain the cytotoxic effects revealed by our results obtained after OECM-1 cells' exposure to both MA-CAD/CAM and MA-3D samples. It has been highlighted that PMMA-based resins' cytotoxicity is greatly influenced by the amount of leachable residual monomers (24). Moreover, several studies outlined that the degree of polymerization, and consequently the residual monomers percent, are strongly influenced by the resin manufacturing technique. More precisely, recent studies revealed that the layer-by-layer polymerization of resins during 3D

printing has been correlated with higher levels of residual monomers compared with milling from highly pre-polymerized blocks (26,27). This is the reason why we aimed to explore the possible cytotoxic effects of both types of PMMA-based resins with applicability in maxillary obturators' manufacturing. However, most of the studies regarding cytotoxicity and biocompatibility of CAD/CAM milled and 3D-printed PMMA-based resins were conducted on human gingival fibroblasts, oral keratinocytes or L929 cells (28). According to our knowledge, there are very few studies focused on investigating possible cytotoxic effects of CAD/CAM milled and 3D-printed PMMA-based resins on the OECM-1 tumoral cells.

Furthermore, in order to a better understanding of the viability and cytotoxicity results, we aimed to expand our explorations at a deeper level, that of the cellular signaling pathways. Therefore we have investigated the possible alterations induced by the two types of PMMA-based resins on the Akt/mTOR pathway and its downstream target, apoptosis, a promising solution in the cancer treatment quest.

Our results revealed an increased expression of GSK3 protein in OECM-1 tumoral cells after exposure to the MA-CAD/CAM samples (Figure 4). For the same samples, our data illustrated a decreased expression of the p53 (Figure 6b) protein and increased levels of JNK, compared to control (Figure 6a).

In the case of incubation with MA-3D dental materials for 24 hours, the level of GSK3a protein expression (Figure 5a) was increased by 38% compared to control. Furthermore, PTEN levels decreased by 32% compared to control (Figure 5b). Moreover, a significant increase in JNK and caspase Bcl-2 expression (by 84% and 85%, respectively, compared to the control) was induced following the exposure for 24 hours to the MA-3D dental materials (Figure 7a and c). In addition, the MA-3D dental materials induced an increase of 72% of

control in the caspase-9 expression level (Figure 7d). Furthermore, the caspase-8 level almost doubled (it increased by 91% of control) after exposure compared with the level of caspase-8 in the control (Figure 7e). Regarding the p53 protein a 16% decrease in protein level from control was recorded after 24h.

Numerous studies highlighted that the Akt/mTOR signaling pathway is involved in the regulation of a series of cellular processes in both normal and tumor cells, playing key roles in proliferation control (29). The activated Akt protein is vital for cell survival (29). As a protein kinase, the active Akt downregulates pro-apoptotic factors and induces the activity of anti-apoptotic effectors by phosphorylating several downstream targets. Akt is able to directly phosphorylate the pro-apoptotic effector, Bcl-2 associated agonist of cell death (BAD), to induce anti-apoptotic signals (29,30), outlining its importance in the cancer molecular landscape. The Akt kinase can also inactivate caspases and the forkhead box protein O1 (FOXO-1). FOXO-1 acts as a transcription factor, regulating the expression of pro-apoptotic genes, such as Bcl-2-like protein 11 (Bim) (29,30). Akt has also been described as an orchestrator of glycogen synthase kinase 3 (GSK-3) activity (31). GSK-3 is a member of the mitogen-activated protein (MAP) kinase family. It has been highlighted that the activated Akt/mTOR signaling pathway induces an inhibitory phosphorylation of GSK3, resulting in the activation of specific transcriptional factors in response to carcinogenesis (31). However, despite the great number of studies of GSK 3 signaling, the orchestration of GSK 3 in cancer progression is still a challenge (31). Taking into consideration the ability of GSK 3 to regulate various proto-oncogenic proteins and checkpoints proteins of the cell cycle, GSK 3 is regarded as a key tumor suppressor (31). Recent evidence outlined that GSK 3 can phosphorylate several tumor factors, such as cyclin D1, c-jun, p65 and

p53, promoting their degradation and preventing their entrance into the nucleus (31). Moreover, it has been demonstrated that GSK 3 inactivation suppressed apoptosis in pre-invasive and invasive oral squamous cell cancer (31). In alignment with all these findings, our results revealed increased expression of GSK 3 (Figures 4, and 5a), in the absence of any Akt activation, triggering molecular events that might explain the observed viability decrements following the OECM-1cells exposure to both types of PMMA-based resins (Figure 1). Furthermore, the increased levels of GSK 3 illustrated by our results (Figures 4, and 5a) may explain the reduced level of p53 expression observed in our study for both types of tested PMMA resins (Figures 6b, and 7b).

As mentioned above, a great number of studies strongly highlighted that the discrete molecular event that underlies the cytotoxic effects of PMMA-based dental resins involves the oxidative stress generation (19). The multifaced ROS is a necessary secondary product of cellular metabolism. However, ROS are regarded as the discreet agents of the complex cellular signaling network (19). Interestingly, malignant cells are able to maintain higher ROS levels compared to normal cells, in order to support their malignant development (32,33). However, excess ROS accumulation can become detrimental even to the survival and proliferation of cancer cells (32,33). Therefore, regarding our study, we might speculate that the exposure of OECM-1cells to the two types of PMMA-based resins triggered a ROS overproduction which negatively orchestrated the Akt/mTOR pathway and, consequently, favored the increased expression of GSK 3, illustrated by our results.

An increasing number of studies highlight the apoptosis image as a key target in cancer chemotherapy (32). Most of the apoptosis mechanism initiation actually, leads to the activation of caspases, the death

executioners. The activated caspases will destroy the cell from within. ms of apoptosis initiation converges on activation of caspases that destroy the cell from within (34). During apoptosis, the active caspase-9 induces the activation of its downstream effector, caspase-3, which will trigger a cascade of molecular events that culminate with cell death (32). The response to intracellular cell death stimuli, the intrinsic apoptosis, is orchestrated by the interplay of the B-cell lymphoma 2 (Bcl-2) family members and their complex interactions with the mitochondrial membrane. Bcl-2 is an integral membrane protein, mainly located on the outer mitochondrial membrane. The Bcl-2 family of proteins include members that exhibit either anti- or pro-apoptotic actions. It has been outlined that the Bcl-2/Bax (BCL-2-Associated X protein) ratio is decisive in determining cell susceptibility to apoptotic death (32). When the outer mitochondrial membrane presents breaches, which might be triggered by oxidative stress, the pro-apoptotic factors such as cytochrome c are released, inducing the initiator caspase, caspase-9, upregulation, which will further activate the downstream caspase cascade (34). However, it has been pointed out that the cytochrome/caspase-9 pathway, activated by stress signals, doesn't seem to be the unique path towards caspase cascade activation. Therefore, Bcl-2 should orchestrate steps upstream of and in addition to the possible ROS-. induced cytochrome c release (34). It has been highlighted that Bcl-2 roles as pro-survival protein is not limited to apoptosis regulation. Being able to bind the autophagic regulator Beclin-1, Bcl-2 has been aside with a key role in autophagy upregulation (34). Autophagy, in its turn, might exert its role as an apoptosis initiator. In alignment with all these findings, in the case of the MA-3D printed samples, our results revealed significantly increased levels of caspase-9, the caspase initiator, and caspase-8 (Figures 7d, and e),

concomitantly with increased expression levels for Bcl-2 (Figure 7c). Moreover, our results also showed strong positive correlations between Bcl-2 expression levels and caspase 9 and 8, respectively, for both types of tested dental resins (Tables 1 and 2; Figures 8-11). All these results might suggest the activation of a detouring caspase-activating signaling pathway, possibly upregulated by Bcl-2, and probably involving the autophagic machinery via Bcl-2 signaling.

It has been pointed out that GSK-3 triggered the heat-shock-induced apoptosis in human neuroblastoma cells. GSK-3 inhibition attenuated the upregulated apoptosis (35). Moreover, there are studies outlining that GSK-3 facilitates caspase-3 activation and apoptosis induced by inhibition of mitochondrial complex I (35). However, the reports of GSK-3 in apoptosis are not consistent. Therefore, the possible correlations between the observed increased GSK-3 levels and the activated apoptosis, illustrated by the increased levels of caspase 9 and 8, in the condition of our study need further investigation.

Taken together, our results showed that the exposure of OECM-1cells to two types of PMMA-based dental resins (CAD/CAM milled- MA-CAD/CAM and CAD/CAM 3D printed – MA-3D printed) induced cytotoxic effects, probably via a residual monomer-induced overproduction of ROS, triggering the decrement of cell viability. Furthermore, exposure to the PMMA-based resins disturbed the Akt/mTOR/GSK-3 signaling pathway compared to the unexposed control cells. Moreover, our results might suggest that the noticed decreased cell viability should be attributed to the activation of apoptosis, via a detouring signaling pathway, possibly orchestrated by Bcl-2 and probably involving autophagy. Autophagy involvement in the molecular landscape of the OECM-1cells behaviour in our study conditions definitely deserves attention and further investigations.

Conclusion

Within the limitations of the present in vitro study, our results surprisingly revealed that exposure of OECM-1 cells to the two types of PMMA-based dental resins (MA-CAD/CAM and MA-3D printed) induced cytotoxic effects and reduced cell viability probably by activation of apoptosis. Due to the experimental limitations of the present study, our data must be considered preliminary. Further in vitro investigations

are needed to validate our findings. Also, further research should focus on a deeper understanding of the molecular aspects of the interactions between the materials used for obturators' manufacturing and cancer cells. However, our results might encourage future research to design resins-based maxillary obturators which, through the polymer chemical composition, should ensure the inhibition of growth and proliferation of the possible residual malignant cells after tumor resection.

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