PROSJEKTOPPGAVE FOR DET INTEGRERTE MASTERGRADSSTUDIET I ODONTOLOGI

CYTOTOXICITY OF ELUATES FROM DENTURE BASE MATERIALS

LONG GIANG NGUYEN

VEILEDERE: MARIT ØILO OG MIHAELA ROXANA CIMPAN

UNIVERSITETET I BERGEN

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Contents

Abstract

Background: Some patients do not tolerate polymethylmethacrylate-based (PMMA) dentures. Polyamide as an alternative to PMMA has, however, not been well documented regarding to biocompatibility and cytotoxicity.
Aim: The purpose of this study was to determine in vitro the effect of eluates from polyamide denture base resin on apoptosis and necrosis.
Materials and methods: Eluates from three auto-polymerized PMMA, an auto-polymerized vinyl polysiloxane, a heat-polymerized PMMA and two polyamide based resin were investigated. The characteristics of apoptosis and necrosis were evaluated by flow cytometry.
Results: The results showed no statistical differences in cell death among the materials.
Conclusion: No definitive conclusion could be drawn based on the results of this study. Further studies are needed in order to make a thorough evaluation of the biocompatibility of polyamide-based denture base materials.

Sammendrag

Bakgrunn: Noen pasienter tolererer ikke polymetylmetakrylat baserte (PMMA) tannprotester. Polyamid er brukt som et alternativ til PMMA, men det mangler fortsatt dokumentasjon med hensyn til biokompatibilitet og cytotoksisitet.
Mål: Hensikten med denne studien var å bestemme, in vitro, effekten av eluater fra polyamid protesebasis resin på apoptose og nekrose.
Materialer og metoder: Eluater fra tre autopolymeriserte PMMA, en autopolymerisert vinyl polysiloksan, en varm polymerisert PMMA og to polyamid-basert resiner ble undersøkt. Kjennetegnene for apoptose og nekrose ble evaluert ved flow cytometri.
Resultater: Resultatene viste ingen signifikant forskjell i celledød mellom de ulike materialene.
Konklusjon: Basert på resultatene av denne studien kan ingen endelig konklusjon trekkes. Videre forsøk er nødvending for å kunne kartlegge biokompatibiliteten til polyamid-basert protese materialer.

Introduction

Dentists face the challenge of constructing better dentures that not only withstand fractures but are also safe, comfortable and more aesthetic pleasing for the patient. Most commonly, removable dentures are made of polymethyl methacrylate (PMMA), also known as acrylic dentures. Even though acrylic is the most common material, it has a few shortcomings. The material has a high fracture incidence (1). A study from 1969 showed that over a million PMMA-dentures were repaired in a year, almost as many as were made (2). The material lacks the ability to withstand tension and compression which can make it break easily. To avoid fractures, the dentures require a certain thickness which can be perceived as uncomfortable for patients. Another weakness with acrylic dentures is that patients and dental technicians can get allergic reactions when exposed to the material in use or during productions (3). Oral reactions often include symptoms such as burning mouth and tongue, erythema, and erosions of the oral mucosa (4). Acrylic dentures have an exceptional resistance to the oral environment, many solvents and UV radiation. But there are still small risks of toxicity and hypersensitivity to the material due to leaching of initiators, oxidation products, and other components in the material (3, 5, 6).

Nylon is a generic name for a group of thermoplastic polymers known as polyamides. Polyamide is a thermoplastic elastic material which means that it deforms permanently when it is heated to a certain temperature and cooled off. Since it is also an elastic material it will deform temporarily under exposure to a certain temperature but then resume its original form at lower temperature. Polyamides have been introduced as an alternative denture material to overcome the disadvantages of PMMA. They were first used in the 1950s, but were not popular as they discoloured quickly and had high absorption of water (2). Polyamide has since then been modified, and is used today as an alternative to acrylic dentures, especially removable partial dentures. There is limited documentation on mechanical and biological properties as well as clinical performance of the modern polyamides.

Denture base materials

There is a constantly ageing population in the world and a report from the United Nations in 2013 estimates that by 2050 over 50 % of the population in developed countries will be over the age of 50 (7). It is likely that many of these people will need prosthodontic treatment in form of partial or full dentures. It is therefore important that this population is given highly aesthetic and functional dentures to increase the quality of life.

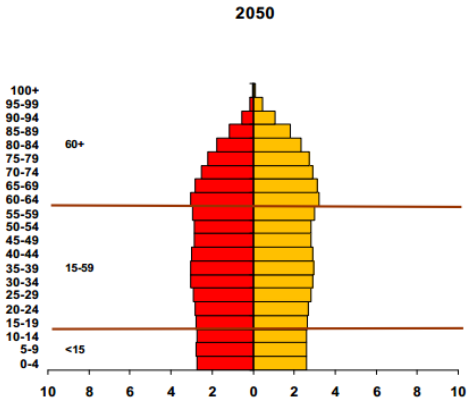

Figure 1. The estimated population pyramid for developed countries in 2015(With permission from United Nations Publications) (7).

It is said that the first dentures were made in Egypt about 2500 BC (8). Since then different materials have been applied for this purpose, such as wood and bone which were very popular because they were inexpensive and easy available. Metal alloys such as Ni-Cr and Co-Cr were used in 1907 as a denture base, but allergy to nickel made it difficult to use. Co-Cr alloys is used widely today both in removable and fixed prosthodontics. Vinyl resins that were used had difficult processing methods and fractures were common. Vulcanite dentures were the first durable and functional dentures, and the first to be mass produced. But because of the dark colour and high absorption of saliva, they were replaced by the more aesthetic pleasing acrylic dentures when Dr. Walter Wright introduced PMMA as a denture base material in 1937. This material is divided in two groups based on the polymerization method:

1) Heat-activated or heat-cured PMMA is prepared by mixing polymethyl methacrylate powder with benzoylperoxide (initiator) and methyl methacrylate liquid. Different heating methods are used to activate the material, such as boiling water or microwave. Upon heating, benzoylperoxide molecules break down to free radicals to initiate the polymerization process.

2) Cold-cured or autopolymerizing PMMA also comes in a powder-liquid form, and is activated through a chemical compound, a tertiary amine such as N,N-dimethyl-p-toluidine, rather than heat. The advantages with cold-cured PMMA is a reduced shrinkage during polymerization, but unreacted monomers can cause unwanted reactions with the tissue (8, 9).

Properties of polyamide as a denture base material

Mechanical properties of denture base materials are usually described as flexural strength or flexural modulus. Compared to PMMA, polyamide has lower flexural strength and higher flexural modulus (10). The polyamide denture material has better fracture resistance than the PMMA materials but lower resistance to deformation. A thermoplastic material is deformable above a specific temperature, but returns to a stable state upon cooling.

Polyamide is produced by a condensation reaction between a molecule with carboxylic (COOH) groups on each end and a molecule with amine (NH_2) groups on each end (Fig. 2).

The polyamide name is often followed by two numbers, indicating the number of carbon atoms in the diacid and the diamine. Its properties are determined by the number of these carbon atoms. Nylon 6,6 is the most common, and it contains 6 carbon atoms in both the diacid and diamine (R=4C, R'=C).

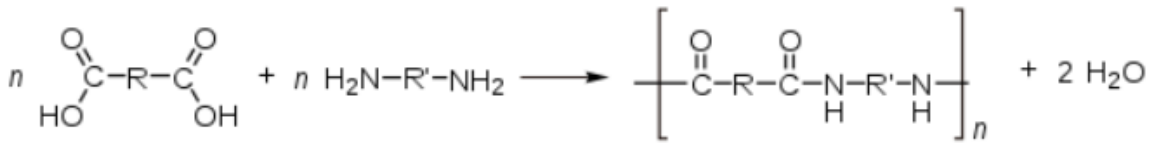

Figure 2. Condensation reaction between a diacid and a diamine molecule to form a polyamide molecule (11).

Because the denture is located in the mouth, it is exposed to a humid environment. In which manner water and other substances are absorbed and released from the material, is important to know in order to understand the materials' dimensional and chemical stability. The water molecules can diffuse between the molecules of the material and force them apart (12). This phenomenon affects the denture stability and form, and water sorption and solubility of denture base materials should be as low as possible (13, 14).

Clinical use of polyamide dentures

The first clinical use of polyamide as denture base material showed many disadvantages. The color changed quickly and the material absorbed colors and water from food. Besides this, the surface of the material was roughened after a short period of usage (15, 16). But it was not until 1971 that the problems were overcome when Hargraves used nylon 12 instead of nylon 6. In addition, Hargraves reinforced the denture base material with glass fibers (2). Nylon 12 is made from a polymerization reaction of laurolactam $(C_{12}H_{23}NO)_n$ monomers which have 12 C-atoms, whereas nylon 6 is made by caprolactam $(C_6H_{11}NO)_n$ monomers (17).

Polyamides can be used for manufacturing complete dentures, partial dentures, or temporary dentures (Figs. 3 and 4). The flexibility of the material may be beneficial for patients with abnormal morphology of the alveolar process which complicates the use of a conventional denture.

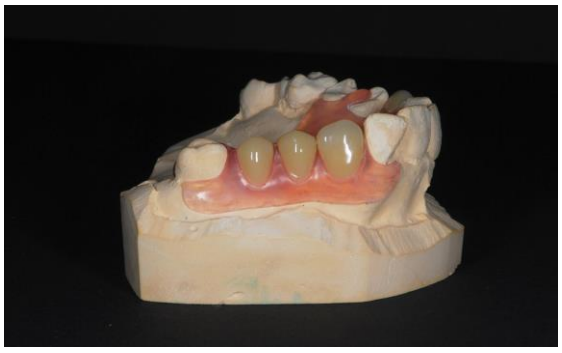

Figure 3. A removable partial denture made of polyamide (With permission from Rune Haakonsen, UiB)

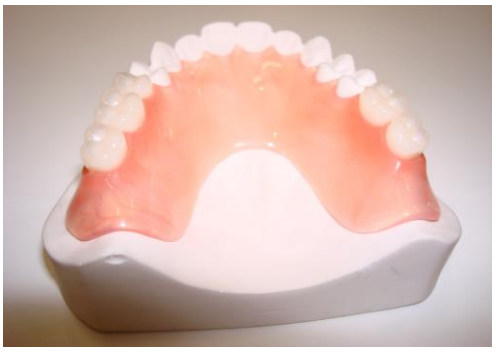

Figure 4. A complete denture made of polyamide (With permission from Heraeus Kulzer®)

The greatest advantage with polyamides is their elasticity, which means that any movements in the oral cavity are easy for the material to adapt to. For dentures covering a large area of soft mucosa this flexibility is, on the other hand, not convenient because the denture will distort during chewing. Since polyamide is soft and light weighted it will also make it more comfortable for the patient and since the color of the material looks like the gingiva, it improves the aesthetics.

Studies have shown that acrylic dentures can give allergic reactions (18) and there are cases that report hypersensitivity to denture materials (19). Polyamide is considered as less allergenic than acrylic dentures and therefore a better alternative for patients with PMMA allergy. There is, however, limited scientific documentation to support this assumption (20). There are only a few case reports and no clinical trials as well as very limited documentation on the mechanical stability, solubility and durability of modern polyamides.

Biocompatibility

Dental materials are constantly in contact with the oral tissue and can sometime induce various tissue damages. A biomaterial is placed in a biological environment and is considered biocompatible when it has the ability to be in contact with a living system without producing adverse effects (21). There are several test methods used to evaluate whether or not a material is compatible, e.g in vitro tests in cell cultures and in vivo tests in animal experiments, or patients. Assessing biocompatibility is a difficult process, and these tests help to understand the biological response of a material, but cannot define whether or not the material is completely biocompatible (22). Even though one test method can show little or no risk of damaging effects regarding a dental material, it is important to remember that there is a large number of patients who will come in contact with the material. Some of these patients may still develop unwanted effects such as allergy or hypersensitive reactions. A material can therefore be individually compatible for one patient but not for another patient (23).

Flow cytometry

Flow cytometry is a method employed to measure and analyze a suspension of cells as they flow in a fluid stream, one by one, through a beam of light (usually laser) (24). The method is reproducible, very accurate and unbiased when analyzing large population of cells. It detects rapidly quantitative and qualitative properties of the cells by measuring optical and fluorescence characteristics. As each cell passes through the beam of light it deflects the light, and *light scattering* occurs. The light is detected by several optical detectors which convert it to an electrical signal for further computer analysis. How much light scattering occurs depends on the size and internal complexity of the cell (Fig. 5).

Light scattered in the forward direction of the laser beam is called forward-scattered light (FSC). FSC is usually collected from approximately 1 to 20 degrees off the laser beam, depending on the size of the cell (25). This signal gives information about the morphology and size of the cell. The information about the internal complexity of the cell is given by the light scattered sideways of the laser beam, called side-scattered light (SSC).

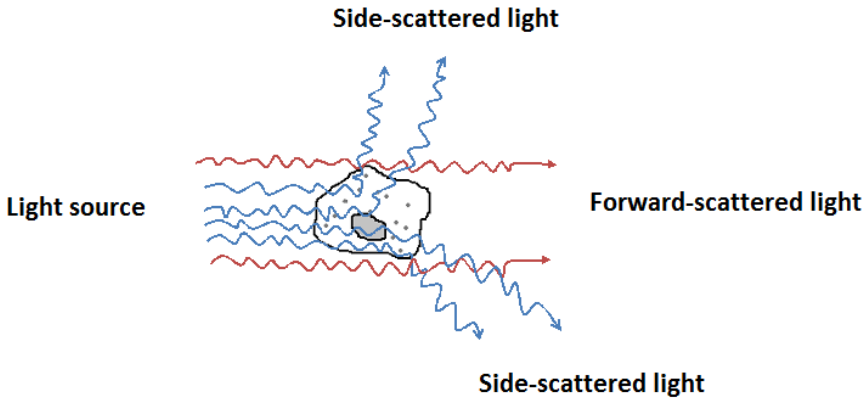

Figure 5. Light scattering properties of a cell.

Fluorescent compounds (fluorochromes) may bind to cellular components such as DNA or specific proteins inside the cell or on the cell membrane. The fluorochrome absorbs the energy from the laser beam causing electrons to excite to a higher energy state. The excited electrons quickly return to their resting state emitting the absorbed energy as photons of light. This is called fluorescence (24).

Cell death

Cell death is a biological event where the cell stops functioning. Two main forms are well studied: apoptosis and necrosis (26). Several classifications of cell death exist and several forms of cell death have been described. Cell death can be classified as apoptotic or necrotic according to morphological appearance, enzymological activity (such as caspases), functional aspect (physiological or pathological) or immunological characteristics (27). Dying cells go through a process which is reversible until they enter an irreversible point, a point of no return. At this state the changes in the cell occur, such as loss of mitochondrial membrane potential (28), caspase activity rises greatly (29), complete permeabilization of outer mitochondrial membrane (30) or exposure of phosphatidylserine (PS) at the cell surface (31). It is important to understand that when a cell undergoes cell death it is not always that it presents all the characteristics to either necrotic or apoptotic (32).

Apoptosis is essential for developing and maintaining cell function. Several typical features are the activation of caspases, chromatin condensation and display of phagocytosis markers, such as PS, on the cell surface. Other features include reduction of cellular volume (pyknosis), plasma membrane blebbing (zeiosis), nuclear fragmentation (karyorrhexis) (27, 33). In cell cultures, where no phagocytes is present, apoptotic cells undergo apoptotic necrosis whereby they gain cell volume (oncosis) and show typical features for necrosis (34).

Necrosis is an unregulated death of cells and is caused by external factors such as trauma or toxins. Unlike apoptosis, this type of cell death is not caused by natural causes and may result in inflammation and be detrimental for the surrounding tissues. Necrosis is morphologically characterized by oncosis, rupture of the plasma membrane, and loss of intracellular contents.

Necrosis is a term commonly used, but it is not specifically defined since there is no consensus on the biochemical changes that identify a necrotic cell (27). Hence the term should be used with caution when classifying a cell as necrotic.

Aim of the study

The aim of the study was to assess *in vitro* the effect of eluates from polyamide denture base resins on cellular viability.

Materials and methods

Denture base materials

Test specimens of three auto polymerized PMMA (Meliodent Rapid Repair, Palacos R and Palacos R+G), one auto-polymerized silicone soft liner (GC Reline Soft), a heat-polymerized PMMA (Ivoclar Vivadent SR Ivocap High Impact Polymer), and two injection molded polyamide based resin (Breflex and Valplast) were investigated (Table 1, Table 2, and Fig. 6).

Table 1. Denture base resins used in the investigation: Producers

Product name	Manufacturer	Batch No.	Material
Meliodent Rapid Repair (MRR)	Haraeus Kulzer GmbH, Hanau, Germany		PMMA
Powder		10SEP166	
Liquid		013142	
Palacos R	Haraeus Medical GmbH, Wehrheim, Germany	71864274	PMMA
Palacos R+G	Haraeus Medical GmbH, Wehrheim, Germany	72104266	PMMA
GC Reline Soft (GC)	GC America Inc., USA	0908281	Vinyl polysiloxane
Ivoclar Vivadent SR Ivocap High Impact Polymer (HIP)	Ivoclar Vivadent AG, Schaan, Liechtenstein	N71950	PMMA
bre.flex transparent, tooth shade B, pink (Breflex)	bredent GmbH & Co.KG, Senden, Germany	733329	Polyamide
Valplast Resin (Valplast)		110636	Polyamide

Table 2. Denture base resins used in the investigation: Properties

Product name	Polymerization	Composition	Melting point
Meliodent Rapid Repair (MRR)	Cold-cure	PMMA N,N-dimethyl-p-toludine	NA
Palacos R	Cold-cure	PMMA N,N-dimethyl-p-toludine	NA
Palacos R+G	Cold-cure	PMMA N,N-dimethyl-p-toludine	NA
GC Reline Soft (GC)	Cold-cure	Ethyl acetate	NA
Ivoclar Vivadent SR Ivocap High Impact Polymer (HIP)	Heat-cure	PMMA, benzoylperoxide	NA
bre.flex transparent, tooth shade B, pink (Breflex)	Thermoplastic	Polyamide	183-187℃
Valplast Resin (Valplast)	Thermoplastic	Polyamide	NA

NA= Not Available

Figure 6. From left: Specimens of HIP, MRR, Palacos, Breflex, and Valplast.

Preparation of test specimen and eluates

The specimens (40 ± 0.2 mm diameter, 2 ± 0.2 mm thickness) were fabricated under aseptic conditions according to the manufacturers' directions and the ISO standard 20795 recommendations (35). Valplast and Breflex were delivered from the manufacturer. Palacos R, Palacos R+G and GC were fabricated in preshaped moulds (Flexistone Plus, LOT: 130702).

Eluates were prepared following the ISO 10993-5 requirements (36). The extraction medium was RPMI-1640 Medium (BioWhittaker, Verviers, Belgium) without supplements which were added to the specimens in 6 cm diameter Costar® polystyrene petri dishes (Fig. 7). The ratio between the mass of the specimens and the volume of extraction medium was 0,4 g/ml

(36). The extraction was performed with agitation at 100 rpm for 24h at 37°C. Inactivated (30 min at 56°C) fetal bovine serum (FBS), Penicilline/Streptomycine/Fungisone (PSF) and L-glutamine (BioWhittaker, Verviers, Belgium) were not added to the extraction medium until exposure to the cells. Eluates were stored at -20°C prior to cell exposure.

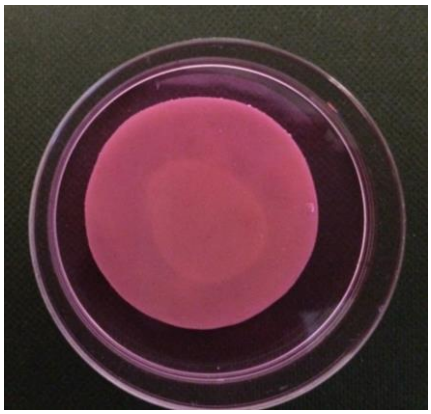

Figure 7. A specimen in extraction medium.

Cell Culture

U-937 human histiocytic lymphoma promonocytic cells (CRL-1593.2; American Type Culture Collection, Manassas, VA, USA) were cultured in suspension in Costar flasks in RPMI-1640 Medium supplemented with 10% heat inactivated (56°C, 30min) FBS, 2% mix of PSF and 1% L-glutamine, and kept in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The cells were maintained at a density between 5 x 10^5 and 1 x 10^6 per ml. The cells were subcultured every 2-3 days.

Exposure of cells to eluates

A number of 5 x 10^5 U-937 cells with a viability higher than 90% (tested by exclusion of 0,2% trypan blue) (Fig. 8) were cultured per well in 1,0 ml of each eluate in 24-well TPP® plates. Prior to addition to the cells, the wells were enriched with the supplements mentioned above. Cells were incubated in eluates for 24 h. Cells treated with 100 UI/ml of human

recombinant tumor necrosis factor alpha (TNF-α) (R&D Systems, Abingdon, UK) and 1mg/ml cycloheximide (Cx) (Sigma Aldrich, Oslo) served as positive controls. Cells exposed to RPMI-1640 Medium incubated under same conditions as the eluates served as negative controls.

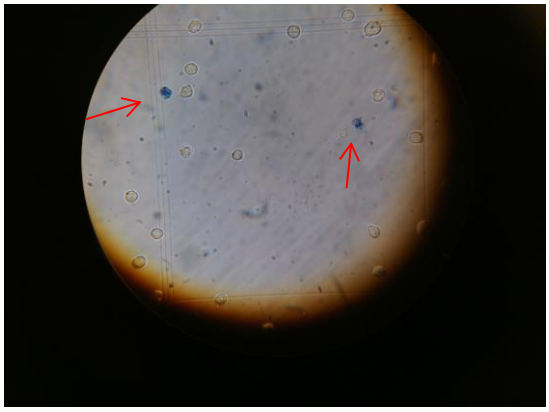

Figure 8. Trypan blue staining. Optical microscopic image of U-937 cells stained with trypan blue dye. Nonviable cells appear as blue (arrows).

Annexin V-FITC and Propidium Iodide assay (PI)

The assay used has been described in detail by Vermes et al. (37) and Cimpan et al.(2000). Translocation of PS from the inner side of the plasma membrane to the outer layer was detected by incubating cells with FITC-conjugated (fluorescein isothiocyante) Annexin V (Nexins Research, Kattendijke, the Netherlands) (Apoptest ^{TM} V-FITC Kit A-700). Cells that lost the integrity of the plasma membrane (i.e. necrotic and secondary necrotic) were detected by propidium iodide (PI). During the assay the cells were kept on ice to arrest further progress through the stages of life towards death. Stained cells were analyzed by a Coulter Epics XL-MCL (Coulter Corporation, Harpender, UK) flow cytometer, equipped with a single argon ion laser. Excitation was at 488nm. Green (FITC) fluorescence was collected at 505-545 nm and red (PI) fluorescence at 605-635 nm. At least 10,000 cells were analyzed per sample. Quadrant setting was based on the negative unstained control. Each experiment run in duplicate was repeated at least three times. Data analysis was carried out with Flowing Software 2 version 2.5.0 (38).

Results

A dotplot of green fluorescence (Annexin V-FITC) versus red fluorescence (PI) showed three separate clusters: viable cells (left, lower quadrant), apoptotic cells (lower, right quadrant) and necrotic plus secondary necrotic cells (upper, right and left quadrant) (Fig. 9).

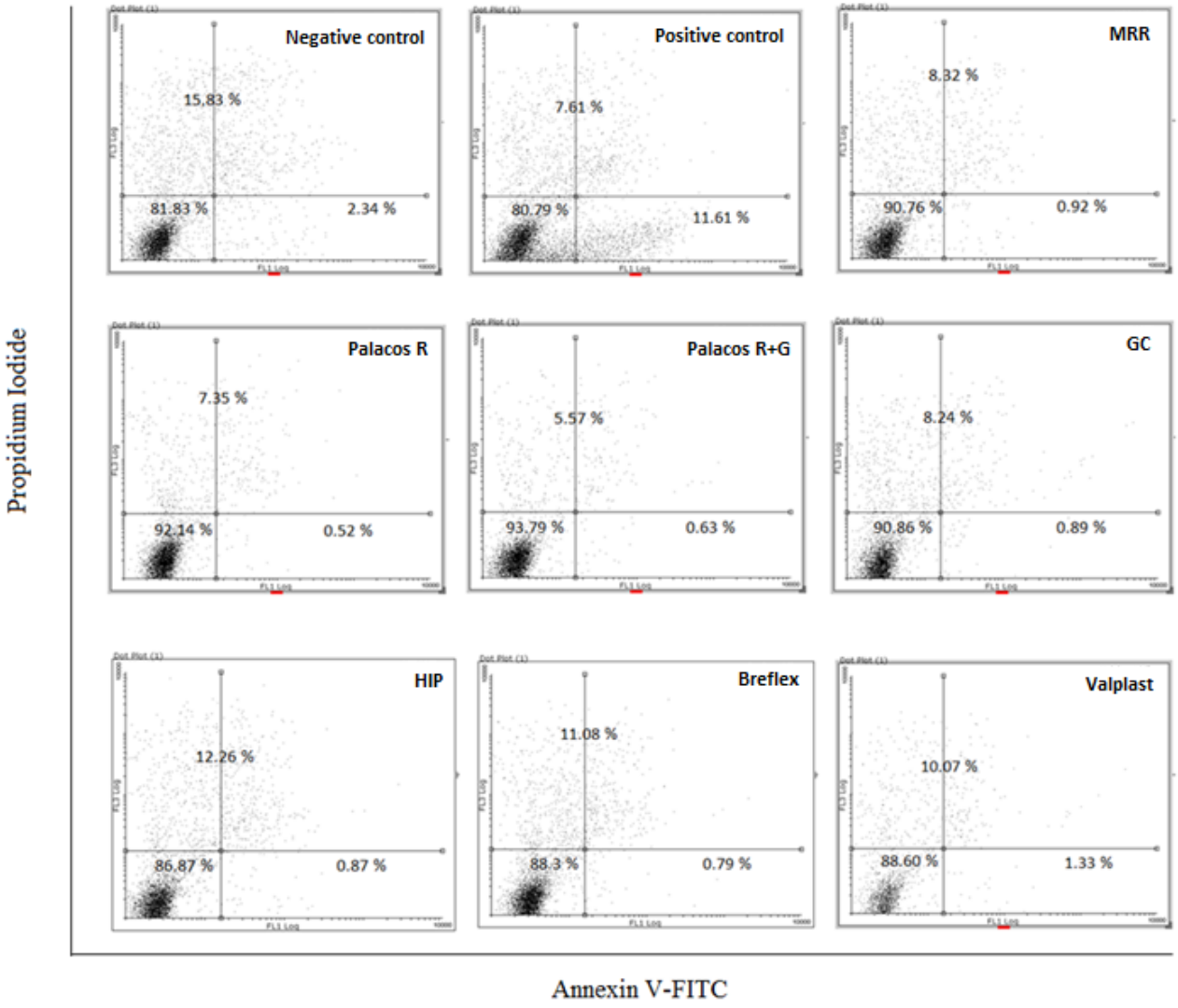

Figure 9. Effect of eluates on Annexin V-FITC/PI staining of U-937 cells for the different materials . 1) Viable cells in left lower quadrant 2) Apoptotic cells in right lower quadrant 3) Necrotic and secondary necrotic in upper right and left quadrant.

The levels of apoptosis and necrosis induced by eluates from the different denture base materials is shown in Fig. 10.

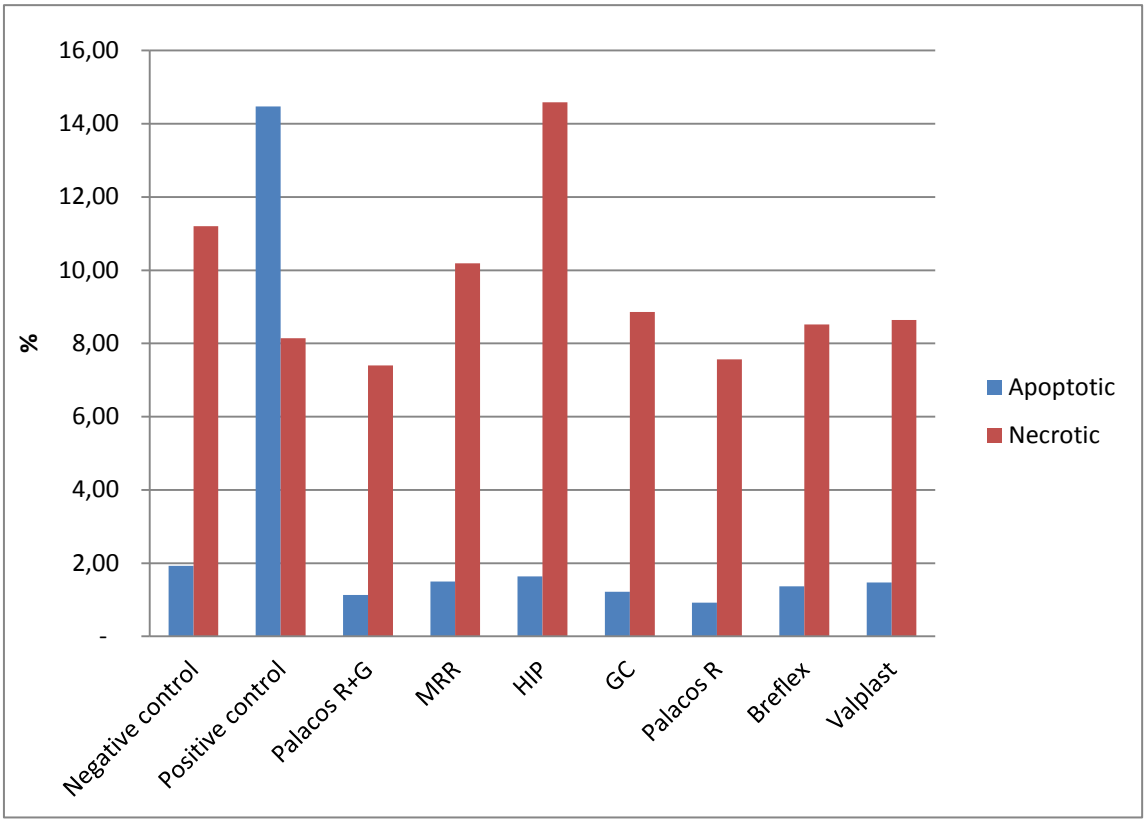

Figure 10. The average percentage of apoptotic and necrotic cell death induced by eluates of 7 different denture base resins.

Eluates from HIP showed higher levels of necrotic cells than the other materials tested. There were no statistical differences in cell death among the tested materials. We cannot say whether the results are significant or not because of the high levels of necrotic cells in the negative control, which means low viability of cells when analyzed by flow cytometry.

Discussion

Permanent cell lines are recommended for toxicity screening of dental materials due to their good reproducibility, genetical and metabolical stability (39). The U-937 permanent cell line was chosen for this study because of its human origin and ability to perform many of the

monocytes' functions. The cells can therefore imitate an immune response to the eluates from the materials.

When detecting cells with flow cytometry, the samples must be analysed immediately after staining with Annexin V-FITC and PI. A disadvantage with this method is that the cells progress further through the stages of life towards death in the period after staining until analysed by flow cytometry. This could result in a lower viability compared to the one tested with trypan blue.

Because of the high levels of necrotic cells in the negative control, the relevance of the results is difficult to interpret. The amount of cell death should have been below 10% in the negative control, as a result the reliability of the measurements was considered low.

There could be several reasons for the high amount of cell death in the control. The cells could have been polluted by external factors but this risk is low. A more likely reason is the fact that the flow cytometer used initially stopped functioning and was replaced by a new instrument. Given the short time left, we did not have the possibility to try different settings and repeat all the measurements with the new instrument. The results obtained with the two flow cytometers differed, and thus, new measurements are clearly needed.

Studies have showed that the most toxic effects of denture base materials occur during the first 24 h of exposure (40). However, reports showed that resin-based materials may still leach cytotoxic components after two weeks (41). The results from this study tested the effect of eluates on cell viability for only 24 h. Long-term exposure may therefore be more useful to determine the toxicity of a dental material (42, 43).

Biological complications may occur when using dentures. Oral candidosis is a common opportunistic infection in denture wearers (44). Candida biofilms show a significantly higher growth on polyamide resin compared with PMMA resin (45). Studies have suggested that the residual monomer found in PMMA resin produces a difference in the material surface charge which can make it capable of reducing adhesion and inhibiting the growth of Candida (46). The surface roughness and the difficulty of polishing polyamide base resin (47) may lead to

bacterial and fungal growth on its surface. Patients wearing polyamide based dentures should be more hygiene conscious than if PMMA resin were used.

There are still lacking studies on clinical use of nylon dentures. Since there is no study that can prove that biological complications will not arise using nylon, it should only be used as a temporarily alternative to acrylic dentures. Permanent use is not recommended since we do not know how this will affect the oral cavity and the patient.

Conclusion

No definitive conclusions could be drawn based on the results of this study regarding the cytotoxicity of the different denture base materials. Further tests are needed in order to obtain a complete set of reliable measurements.

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