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Denture base resins biocompatibility testing *in vivo*

Ispitivanje biokompatibilnosti akrilata za izradu zubnih proteza *in vivo*

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Abstract

Background/Aim. The wearing of acrylic dentures is the cause of the inflammatory reaction of the oral mucosa. The aim of this study was to investigate the response of rat tissues to subcutaneous and intramuscular implantation of different acrylic samples, by histopathological analysis of the tissue. **Methods.** The study included two samples of hard and three samples of soft acrylic resins (heat and cold polymerized), that were subcutaneously and intramuscularly implanted in rats tissues. Implantation tests were designed to test the biological response of the surrounding tissue to the tested materials after their application for the period of two weeks and the period of four months. **Results**. After two weeks, regardless of the type of implantation, histopathological analysis showed an acute inflammatory response. There was an intense hyperplasia of inflammatory cells, multiplication of connective tissue as well as formation of many new blood vessels. The highest level of inflammatory changes was observed after the application of cold-polymerized resins. A lower intensity of inflammation in the case of heat polymerised resin was the result of its more complete polymerization. After the second observation period, fibrotic capsules were formed around the implanted samples indicating a chronic course of the inflammatory process. Less visible signs of inflammation and chronicity of the processes indicate that with time, i.e. with the length of the observation period, reduces inflammation. **Conclusion.** The **s**ubcutaneous and intramuscular implantation of acrylic resins material samples led to inflammatory response whose intensity was decreased over time. Heat polymerized resin was a biologically more acceptable in comparison to the cold polymerized acrylates.

Key words:
dentures; acrylates; biocompatible materials; rats.

Apstrakt

Uvod/Cilj. Nošenje akrilatnih proteza uzrokuje inflamatorne reakcije oralne sluzokože. Cilj ovog istraživanja bio je da se ispita odgovor tkiva pacova nakon potkožne i intramuskularne implantacije različitih akrilatnih uzoraka pomoću patohistološke analize tkiva. **Metode.** U studiji su korišćena dva uzorka tvrdih i tri uzorka mekih akrilatnih smola (toplo i hladno polimerizovanih) koji su supkutano i intramuskularno implantirani u tkivo pacova. Implantacioni testovi su osmišljeni tako da se ispita biološki odgovor okolnog tkiva na testirane materijale nakon njihove primene u periodu od dve nedelje i periodu od četiri meseca. **Rezultati**. Posle dve nedelje, bez obzira na vrstu implantacije, patohistološka analiza pokazala je akutni inflamatorni odgovor. Došlo je do intenzivne hiperplazije inflamatornih ćelija, umnožavanja vezivnog tkiva kao i formiranja brojnih novih krvnih sudova. Najviši nivo upalnih promena primećen je nakon aplikacije hladno polimerizovanih akrilatnih smola. Niži intenzitet inflamacije kod uzoraka toplo polimerizovanih smola rezultat je njihove potpunije polimerizacije. Nakon drugog perioda posmatranja uočeno je formiranje fibroznih kapsula oko implantiranih uzoraka što ukazuje na hroničnan tok upalnog procesa. Manje vidljivi znaci zapaljenja ukazuju na transformaciju zapaljenske reakcije u hronični oblik u kojoj se vremenom, odnosno tokom dužeg perioda posmatranja samo zapaljenje smanjuje. **Zaključak**. Supkutana i intramuskularna implantacija akrilnih uzoraka dovela je do inflamatornog odgovora čiji je intenzitet smanjen tokom vremena. Toplo polimerizovani akrilati su biološki znatno prihvatljiviji u odnosu na hladno polimerizovane akrilate.

Ključne reči:
zubna proteza; akrilati; biokompatibilni materijali; pacovi.

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Introduction

Wearing of denture plates is very frequently the cause of inflammatory reactions of submucose of the oral cavity [1, 2]. Hypersensitivity to acrylates was observed in almost 17% of patients wearing dentures [3].

Adverse effects related to acrylates are, in most cases, of local character and may be presented in form of cheilitis and stomatitis, stinging and burning in the mouth, painful sensations of different intensity and candidiasis [4–8]. Allergic reaction to acrylic denture may occur in more severe form such as erythema multiforme [9]. Potential toxicity of temporary acrylic dentures was well-documented. Contact stomatitis in children caused by wearing orthodontic appliances was described in clinical practice as well [10].

The above mentioned changes are more frequent in patients with already infected, inflammed submucose of the oral cavity damaged with different drugs or vomitting [11, 12]. Some regions of the oral cavity are particularly sensitive to irritating effects of acrylic dentures [13]. Apart from being placed to endure additional strain, zones with keratinized epithelium represent places less sensitive to effect of harmful components of acrylates as well [14].

Intense gingival inflammation under acrylic veneers of bridges, may, among other things, be explained by porousness and superficial roughness as the result of a great abrasion of acrylic material. Regarding the complexity of the potential clinical biocompatibility investigation of acrylic materials used to manufacture dentures, it is easier to analyse tissue reaction to acrylic materials after implantation of the samples in tissues of experimental animals. Such studies comparing effects of different types of commercially available acrylic materials *in vivo* conditions has not been performed so far.

The aim of the study was to perform pathohistological analysis of the tissues after subcutaneous and intramuscular implantation of samples of different acrylic materials.

Methods

Tested material

The tested material included two hard and three soft acrylates used in prosthodontic dentistry for construction and readaptation of mobile dental restorations. Cold and hot polymerized acrylates were used in the study (Table 1).

Parallelepiped shaped material samples with rounded edges 1 × 2 × 3 mm were made. Upon polymerization the samples were polished using standard procedure in order to avoid mechanical irritation during the implantation in tissue of experimental animals.

According to the type of the tested materials the samples were divided into five experimental groups (G1-G5), each of which were further subdivided into two groups depending on the place of the implantation (subcutaneous and intramuscular). Each experimental group consisted of twelve samples (n = 12), six samples for subcutaneous and six samples for intramuscular implantation.

A pink wax (Cavex, Holland BV) sample with identical shape and dimensions was made as a negative control for each implanted sample of the tested material (n = 60). Samples were a combination of paraffin, microwax and beeswax and its neutral effect on tissues was previously experimentally proven [17].

All of the tested samples were desinfected with 70% ethanol and rinsed with saline (0.9% NaCl). The samples were stored in sterile Petri dishes at room temperature untill implantation. Immediately before implantation, they were removed to Petri dish with sterile saline (no more than 60 minutes).

Experimental animals

Laboratory Wistar male rats, 10 to 12 weeks old and 180–200 g of average weight were used in the experiment. Twelve animals were used for each of the tested materials (n = 60).

Table 1

Tested acrylic materials

Tested material	Experimental group (G)	Manufacturer	Acrylic type	Content	
				powder	liquid
Bosworth Trusoft	G1	HG Bosworth Company USA	soft cold polymerized acrylate	poly (ethyl methacrylate)	ethyl alcohol, butyl benzyl phthalate
Lang Flexacryl	G2	Lang Dental MFG.Co. USA	soft cold polymerized acrylate	poly (ethyl methacrylate)	n-buthyl methacrylate
Lang Immediate	G3	Lang Dental MFG.Co. USA	soft cold polymerized acrylate	poly (ethyl methacrylate)	methyl methacrylate
Triplex Cold	G4	Ivoclar Vivadent, Lichtenstein	hard cold polymerized acrylate	poly (methyl methacrylate)	methyl methacrylate, ethylene glycol dimethacrylate
Triplex Hot	G5	Ivoclar Vivadent, Lichtenstein	heat polymerized acrylate	poly (methyl methacrylate)	methyl methacrylate, ethylene glycol dimethacrylate

The animals were healthy and acclimatized to laboratory environment and standard laboratory nutrition. They were followed for the behaviour changes, disease onset and weight loss to eliminate potential irregularity that will affect plausibility of the obtained data.

Experimental investigations on animals were approved by the Ethics Committee of the Faculty of Medicine in Niš (number 01-2066-1).

Experimental design

Implantation tests were designed for examining biological response of the surrounding tissue to the tested materials upon their application (ISO 10994-6: 2007) [18].

All animals were operated on under general anesthesia. Premedication included the application of atropin sulphate (Verofarm, Russia) and diazepam (Galenika, Serbia) in a dose of 0.2 mg/100 g of body weight. General anesthesia of 30–60 minutes was administered intraperitoneally with 0.3 mL of 10% Ketamidor® (Richer Pharma A.G., Austria).

Anesthetised animals were placed in prone position on a special wooden framework. The operating field was prepared by removing hairs on interscapulary portion of the back and both thighs.

The implantation region was rinsed with povidone iodine. The implantation procedure was performed using a sterile needle 4/18. The samples were subcutaneously implanted in the interscapulary portion of the back. The sample of the tested material was implanted on the left side of medial dorsal line and the sample of sterile pink wax was implanted on the right side. The sample of the tested material was implanted in *m. gastrocnemius* of the left leg of the experimental animal, while the sample of the pink wax was implanted in the muscle on the opposite side. The wounds were healed with povidone iodine and left to heal spontaneously.

No antibiotic protection of experimental animals was performed. Postoperative recovery was monitored every day, and there were no signs of infection.

The two-week observation period and four-month observation period were designed. After each of the observation periods three animals from each experimental subgroup were sacrificed.

Euthanasia of experimental animals was performed by exanguination of the left ventricle and extirpation of complete blood. Changes in the subcutaneous tissue of the experimental animal after the four-week implantation period were both macroscopically and microscopically observed.

Preparation of samples for microscopic analysis

Tube-like portions of subcutaneous and muscular tissue where resins tested had been implanted were taken as samples for analysis. The samples of the implanted materials were carefully separated from the tissue by twizzers and fixated in 100% formalin. The material was further dehydrated in growing concentrations of ethanol (from 50% to absolute). Upon xylol illumination the material was put in paraffin molds. Tissue blocks molded in paraplast were cut on microtom (LKB Broma, Sweden), (1.5 μm) and stained using classical method – hematoxylin & eosin (HE) and special method for staining of collagen fibres – trichrome staining according to Masson.

The stained preparations were analysed histopathologically on the image analysis system Lucia 3.2 G (Laboratory Imaging, the Czech Republic) on microscope NU-2 (Carl Zeiss, Germany).

The histomorphological analysis of tissues was designed for all samples of subcutaneous and muscular tissue that were in contact with the tested material and negative control for both observation periods.

The evaluation of results by microscopic analysis was performed on the basis of the presence of inflammatory reactions and tissue fibrosis, number and distribution of inflammatory cells and the existence of degenerative changes as well as potential necrosis and destructive changes in capillary walls (Table 2).

Results

After the two-week implantantion period it was noticed that the implantation of Lang Immediate sample material caused macroscopically apparent changes on subcutaneous tissue in the form of mild erythema and local hemorrhage (Figure 1). There were no local macroscopic changes on the implantation site of control samples and other tested materials.

Table 2

Evaluation of a degree of inflammatory reaction [19]

Degree of inflammatory reaction	Score	Changes in the surrounding tissue
Slight reaction	0	Formation of fibrous capsule with sparce inflammatory cells.The blood vesels are of small calibre with visible endothelial cells.
Mild reaction	1	Presence of fibrous capsule with lower number of lymphocytes and plasmocytes. Low proliferation of conective tissue was followed by spherical blood vessels without congestion.
Moderate reaction	2	Formation of fibrous capsule with presence of macrophages, polymorphonuclears, lymphocytes and plasmocytes. Congestion of blood vessels was not noted.
Intensive reaction	3	Presence of a large accummulation of polymorphonuclears lymphocytes, plasmocytes, macrophages, giant cells of foreign body type and capillaries with prominent congestion.

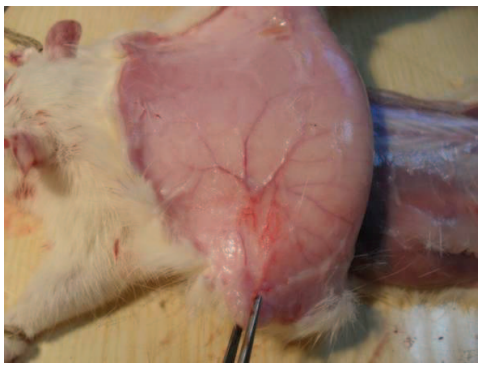

Fig. 1 – Changes in subcutaneous tissue observed at the implantation site of Lang Immediate sample.

Pathological analysis of subcutaneous tissue showed acute inflammatory reaction to the presence of the tested materials. It included intesive hyperplasia of inflammatory cells (polymorphonuclears, lymphocytes, plasmocytes and macrophages), duplication of connective tissue as well as formation of a great number of new blood vessels. Fusion of macrophages led to formation of giant cells as the response to the presence of a foreign body.

The highest degree of inflammatory changes were observed in cold polymerized acrylates (Figure 2). The presence of Triplex Hot sample caused low intensity inflammatory changes in relation to other tested materials which may be attributed to its more complete polymerization (Figure 3).

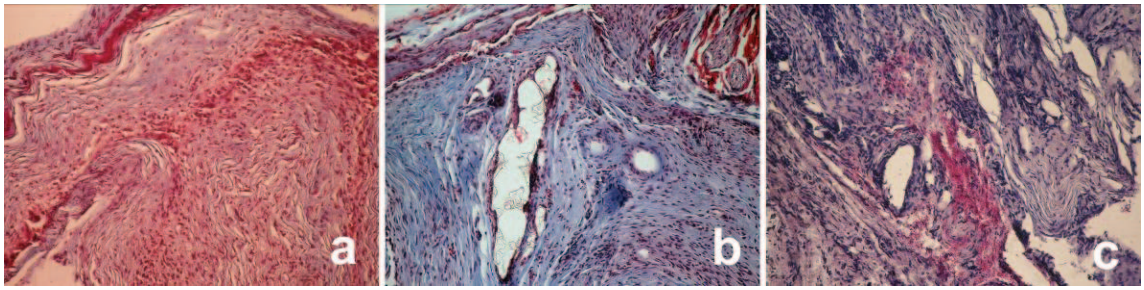

Fig. 2 – Application of samples in subcutaneous tissue of experimental animal after two weeks caused the occurrence of the mild degree fibrosis, hyperplasia of inflammatory cells and formation of new blood vessels: a) Bosforth Trusoft; b) Lang Flexacryl; c) Triplex Cold (Trichrome staining according to Masson, ×100).

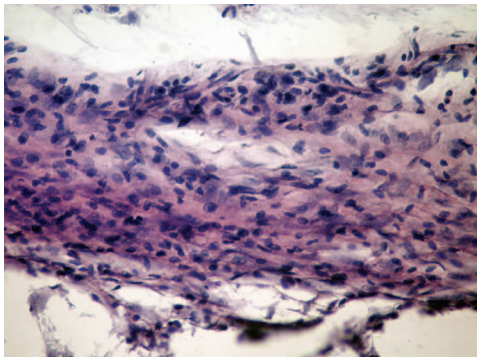

Fig. 3 – After two weeks, subcutaneous application of Triplex Hot sample caused proliferation of connective tissue cells and fibrosis (HE; ×200). HE – hematoxylin eosin.

There was no inflammatory reaction on the site of subcutaneous implantation of control sample, which eliminates mechanical trauma during the application as the cause of occurrence of the above mentioned inflammatory reaction.

After a four-week observation period,-there were no macroscopic changes at the implantation site of acrylic materials in all tested experimental groups.

Fibrous capsules were formed around the implanted samples as the result of the presence of material in the subcutaneous tissue. The hyperplasia degree of inflammatory cells was lower in comparison to the first observation period. Tissue fibrosis with lower number of connective tissue cells indicated chronic course of inflammatory process (Figure 4). Less prominent inflammatory signs and chronic course showed reduction of inflammation over the observed period of time.

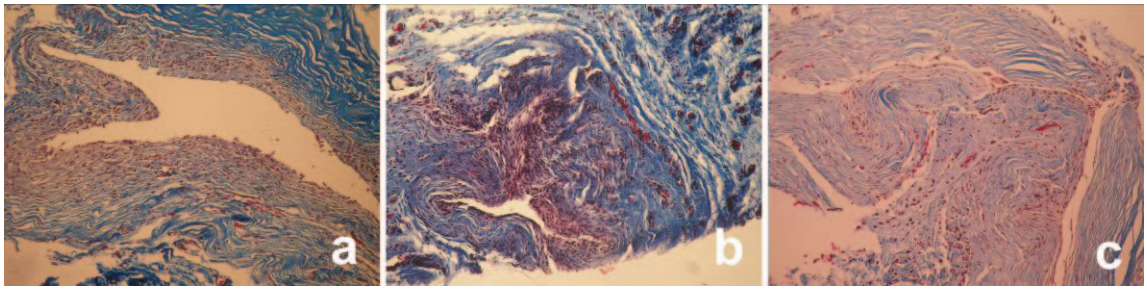

Fig. 4 – After four months of the subcutaneous implantation of material samples focal tissue fibrosis occurred with slight inflammatory infiltrate: a) Lang Immediate; b) Triplex Cold; c) Triplex Hot (Trichrome staining according to Masson, ×100).

Implantation of acrylic samples in *m. gastrocnemius* of experimental animals during the two-week observation period caused no macroscopic changes of the tissue. Histopathological analysis of the surrounding muscle tissue after removal of material samples showed mild to strong inflammatory reaction, hyperplasia of connective inflammatory cells, intramuscular proliferation of connective tissue and a great number of newly formed blood vessels in the tested materials of all experimental groups (Figures 5 and 6).

Formation of fibrous capsule around the application site represented the result of a four-month implantation of the tested material samples on macroscopic level. The tissue fibrosis was from mild (Bosforth Trusoft, Lang Flexacryl, Triplex Cold, Triplex Hot) to moderate degree (Lang Immediate). Pathohistological findings of all tested materials confirmed domination of connective fibres in relation to inflammatory cells, suggesting chronic inflammation, that is, a scar formation in muscle tissue (Figure 7).

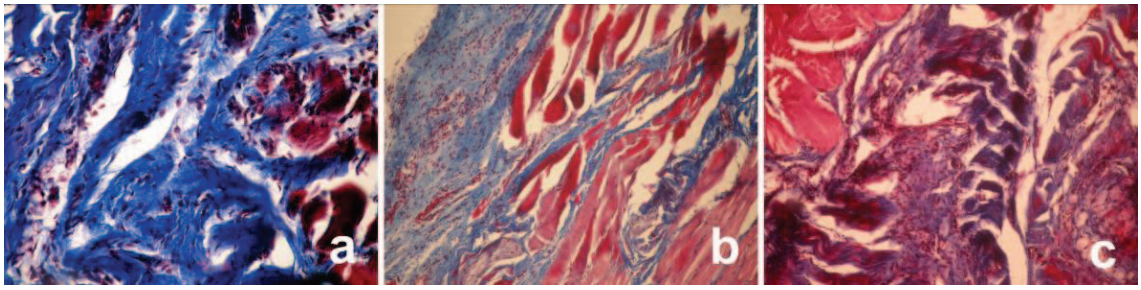

Fig. 5 – After two weeks of the implantation period of material samples Lang Flexacryl (a) and Lang Immediate (b) hyperplasia of giant cells of foreign body type and intensive proliferation of connective tissue were observed. Fibrous reaction with numerous newly formed blood vessels occurred around hollow spaces with Triplex Cold sample (c) (Trichrome staining according to Masson, ×200).

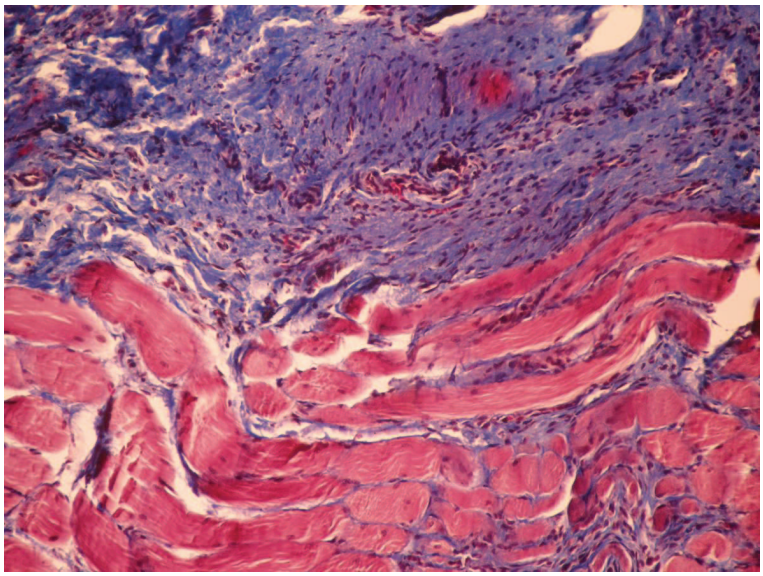

Fig. 6 – A two-week implantation period of Triplex Hot sample caused proliferation of connective tissue cells and intensive fibrosis in muscle tissue (Trichrome staining according to Masson, ×100).

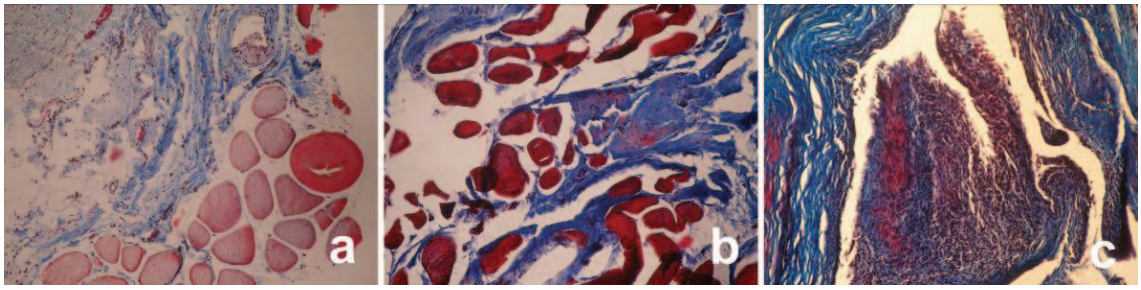

Fig. 7 – The mild degree fibrosis was present in muscle tissue after a four-month implantation of Lang Flexacryl sample (a) and Triplex Hot (b) sample, while the moderate degree fibrosis was present in Lang Immediate sample (c) (Trichrome staining according to Masson, ×200).

Inflammation caused by the presence of implantes acrylic samples reduced over time, that is, along with the duration of the observation period. Implantation of control samples of pink wax caused no significant changes in subcutaneous tissue and muscle tissue in the whole observation period (Table 3).

Table 3

The average values of the prominence degree of inflammatory reaction of tissue after the subcutaneous and intramuscular implantation of acrylic samples

Experimental group (G)*	Subcutaneous implantation		Intramuscular implantation	
	after 2 weeks	after 4 months	after 2 weeks	after 4 months
G1	3	1	2	1
G2	3	1	3	1
G3	3	1	2	2
G4	3	1	2	1
G5	2	1	1	1
Control group	0	0	0	0

***see Table 1**

Discussion

The test of tissue reaction to the implanted material (ISO 10994-6: 2007) has no direct implication on clinical application [18]. However, immediate contact of implanted material and tissue offers a more precise picture of a body reaction to its presence. Subcutaneous implantation turned out to be an efficient method for the examination of biological features of dental materials [19, 20]. The reaction of subcutaneous tissue may be considered analogous to that of submucous tissue regarding their unique histological form. On the other hand, implantation of dental materials in muscle is considered to be a less sensitive method for examination of biocompatibility of dental materials, but the obtained data certainly contributes to the analysis of tissue reaction to acrylates.

Samples of acrylic materials and control samples of pink wax were implanted in the subcutaneous tissue and muscle of rats during the two-week period and four-month period. Changes in the tissues of experimental animals after removal of material samples may be considered analogous to the tissue reaction in direct contact with the denture plates. The disadvantage of the applied test is reflected in the failure to identify the influence of salivary flow and its buffering capacity on the tissue reaction in contact with acrylic material.

Histopathological analysis of subcutaneous tissue showed acute inflammatory reaction after removal of material samples. Intensive hyperplasia of inflammatory cells or granulomatous reaction, duplication of connective tissue as well as formation of numerous new blood vessels were the results of the two-week long presence of acrylic material. Fusion of macrophages led to the development of giant cells as the response to the presence of a foreign body. A more intensive reaction of subcutaneous tissue to the implantation of cold polymerized acrylates represented further evidence of its biological inferiority as compared to heat polymerized acrylate. The obtained data may be explained by greater porousness and superficial adherence of materials as well as greater amount of non-polymerized potentially toxic substances in samples of soft acrylates and Triplex Cold in relation to Triplex Hot. Findings of Kallus [1, 2] also confirmed greater granulomatous reaction of subcutaneous tissue of rats to the presence of cold as compared to hot polymerized acrylate. The results of this study did not indicate significant changes in inflammatory reaction of tissue to the presence of different types of soft acrylic materials.

After the second observation period fibrous capsules formed around implanted samples as the result of the presence of material. Less prominent inflammatory signs and chronic course of the process showed inflammation reduction over the period of time, which is in accordance with the findings of Kallus [2] and Zmener [19].

The presence of Triplex Hot sample caused low intensity inflammatory changes in relation to other tested materials, which may be attributed to its more complete polymerization [21]. Analysing different materials for denture base Ebadian et al. [22] indicated higher biocompatibility of polymerized poly (methylmethacrylate) (PMMA) in comparison to Co-Cr alloys after implantation in buccal vestibulum of dogs.

Pathohistological analysis of muscle tissue surrounding the tested acrylic materials that were removed after the first observation period showed moderate to strong granulomatous inflammatory reaction, hyperplasia of connective inflammatory cells, intramuscular proliferation of connective tissue and a great number of newly formed blood vessels. The most intensive inflammatory response was noticed in soft acrylic material Lang Flexacryl and solid cold polymerized Triplex Cold. After implantation of the Lang Flexacryl sample hyperplasia of giant cells of a foreign body type in muscle tissue was observed. Implantation of Bosforth Trusoft and Lang Immediate samples led to moderate proliferation of young connective tissue in the muscle and duplication of inflammatory cells.

The presence of the hot polymerized Triplex Hot samples in muscle tissue led to the proliferation of connective tissue cells and intensive fibrosis, and reaction had chronic course from the very beginning. In accordance with this study, Stinson [23] showed that implantation of PMMA sample in gluteal muscles of rats led to formation of fibrous capsule over the period of time. Dillingham et al. [24] found mild toxicity of PMMA after it had been implanted in paravertebral muscle of a rabbit. Biocompatibility of PMMA based materials was proved after their implantation in the bone as well [17].

Formation of fibrous capsule surrounding the application site represented the result of a four-month implantation of the cold polymerized material samples on microscopic level. The tissue fibrosis ranged from the mild (Bosforth Trusoft, Lang Flexacryl, Triplex Cold, Triplex Hot) to the moderate degree (Lang Immediate). The histopathological findings of all tested materials confirmed domination of connective fibres in comparison to inflammatory cells, indicating chronic type of inflammation. Inflammation induced by the presence of implanted acrylic samples reduced over the observation period.

Histopathological analysis of the prominence degree of inflammatory reaction of tissue that was in immediate contact with the tested material sample showed more prominent

inflammatory reaction in the subcutaneous implantation as compared to the intramuscular one in all tested experimental groups of acrylic materials. Control samples caused no inflammation of the surrounding tissue and the occurrence of mild fibrosis in few of them was probably the result of mechanical tissue damage during the implantation procedure.

Cold polymerized acrylates induced the most intensive granulomatous reaction after both implantation procedures, which is in accordance with the results of examination of their cytotoxic effect [25–27]. The results of this study are in positive correlation with the findings of *in vitro* studies that proved stronger cytotoxic effect of soft and hard cold polymerized acrylates as compared to hot polymerized acrylates [28–31].

Conclusion

The subcutaneous and intramuscular implantation of the samples of the tested acrylic materials led to acute inflammatory reaction which become chronic over time. Heat polymerized acrylate showed the least proinflammatory effect. Therefore, the authors recommend heat polymerited acrylates as the material of choice for construction and readaptation of dentures.

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